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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/08 | A1

(11) International Publication Number: WO 99/38953

(43) International Publication Date: 5 August 1999 (05.08.99)

(21) International Application Number: PCT/CA99/00069

(22) International Filing Date: 28 January 1999 (28.01.99)

(30) Priority Data:

60/072,858	28 January 1998 (28.01.98)	US
60/072,877	28 January 1998 (28.01.98)	US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DEVELOPMENT OF REGULATORY CELLS AS A MEANS FOR TREATING AUTOIMMUNE DISEASE

(57) Abstract

A population of mammalian T-cells enriched in cells which have a cytokine production profile characteristic of Th2-like cells (IL-4 production, etc.) is produced by culturing T-cells, including thymocytes, in a conditioned medium (CM), which is prepared by culturing mammalian cells such as peripheral blood cells in the presence of plant mitogens. The proliferation and differentiation to single positive T-cells is promoted by the presence of IL-4 in the culture medium. The enriched population of IL-4 producing T-cells according to the invention show potential use as a source of cells for cell therapy in treating IL-4 response related disorders including certain autoimmune conditions such as diabetes mellitus.

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DEVELOPMENT OF REGULATORY CELLS AS A MEANS FOR TREATING AUTOIMMUNE DISEASE

FIELD OF THE INVENTION

This invention relates to processes for the production of selected T-cells. More specifically, the invention relates to processes for producing selected subpopulations of T-cells having desired cytokine profiles, and uses of such cells, for example in cell therapy.

BACKGROUND OF THE INVENTION

T-cells are a major form of lymphocytes and typically constitute a critical component of the mammalian immune system. In this application, the phrase "T-cells" includes both mature and immature T-cells. Immature T-cells (sometimes called thymocytes) are located in the thymus. While located in the thymus, immature T- cells are exposed to numerous growth factors secreted by thymic stromal cells. Immature T-cells also interact directly with thymic stromal cells during development, and such interaction is considered necessary to T-cell development in the thymus.

Immature T-cells undergo numerous changes while maturing in the thymus. Very immature T-cells lack detectable cluster of differentiation (CD) molecules 4 and 8, and are therefore described as $CD4^-$ $CD8^-$ (double negative) cells. During early T-cell development double negative (DN) cells undergo rearrangements in genes encoding cell surface molecules. Following this gene rearrangement, the majority of surviving T-cells begin expressing both CD4 and CD8 and are known as $CD4^+$ $CD8^+$ (double positive) cells. Double positive (DP) T-cells undergo a period of proliferation, which is followed by another genetic rearrangement to produce genes encoding a functional T-cell Receptor (TcR). Those developing T-cells which have undergone productive genetic rearrangements and survived to this point then enter the second major phase of development in the thymus, namely positive and negative selection.

While in the thymus, developing T-cells undergo selection which normally ensures that mature T-cells will recognize self MHC associated with foreign antigen. Thymic stromal cells, including thymic epithelial cells, are believe to play an important role in T-cell selection by providing high levels of self-MHC to which the developing T-cells may bind. The current understanding of this process is that during positive selection of T-cells in the thymus, the differentiation of DP cells into SP cells requires binding of the TcR and CD4 or CD8 co-receptors on DP thymocytes to antigenic peptide-MHC complexes on thymic epithelial cells. Those T-cells which do not bind self MHC, or which bind self antigens with high affinity ("self reactive T-cells"), generally undergo apoptosis and die. Developing T-cells which survive positive and negative selection in the thymus generally develop into either $CD4^+$ or $CD8^+$ single positive (SP) cells.

In their mature form, $CD4^+$ T-cells are MHC class II (MHC II) restricted, and $CD8^+$ T- cells are MHC class I (MHC I) restricted. The mechanism controlling the choice of a $CD4^+$ or a $CD8^+$ fate for developing T-cells is poorly understood. One current model suggests that TcR specificity for MHC class I or class II molecules may determine the lineage commitment of DP thymocytes into either $CD4^-CD8^+$ or $CD4^+CD8^-$ SP thymocytes by the down-regulation of CD4 or CD8 surface expression, respectively. Alternatively, a stochastic/selection model suggests that DP thymocytes may indiscriminately terminate synthesis of either CD4 or CD8, and that the subsequent maturation of DP thymocytes is dependent on the matched TcR and co-receptor specificity.

It is known in the art that treatment with phorbol 12-myristate, 13-acetate (PMA) plus ionomycin can stimulate DP thymocyte differentiation into $CD4^+CD8^-$ and/or $CD4^-CD8^+$ SP thymocytes without TcR engagement, and this differentiation is dependent on the duration and extent of stimulation. Phorbol esters such as PMA are believed to act by activating protein kinase C, an intermediate in the normal signal transduction pathway by bypassing the need for specific cell-surface receptor binding.

Two major functional types of T-cells are T-helper (Th) cells, and T-cytotoxic (Tc) cells. In general, Th cells are $CD4^+$ $CD8^-$ and Tc cells are $CD4^-$ $CD8^+$. However, these categorizations are not absolute and both $CD4^+$ Tc cells, and $CD8^+$ Th cells have been reported. Current understanding suggests that Tc cells are activated by binding to an antigen-MHC complex presented by an altered-self cell in the presence of IL-2. Activated Tc cells are effector cells which cause the lysis of cells presenting an antigen- MHC complex bound by the activated Tc cell.

Tc cells tend to secrete fewer cytokines than Th cells do. The most common cytokine secreted by Tc cells is IFNγ. However, a subtype of Tc cells, called Tc2 cells, has been reported to secrete IL-4.

Th cells are important to both humoral and cell-mediated immune responses. The current literature on Th cell activation suggests that Th cells are activated by the interaction of the TcR-CD3 complex on the Th cell surface with an antigen-MHC II complex on an antigen presenting cell (APC). This interaction is believed to trigger a series of events within the Th cell which result in cell proliferation, cell-surface receptor expression, and cytokine secretion. Activated Th cells may differentiate into memory cells or effector cells.

Th cells may belong to either the Th1 or Th2 subset of Th cells. The subsets Th1 and Th2 are best characterized by their cytokine production profiles. Th cells belonging to the Th1 subset ("Th1 cells") secrete interleukin (IL)-2, interferon (IFN)-γ, and tumour necrosis factor (TNF)-β. IL-2 may be necessary to T-cell activation. Th1 cells promote cell-mediated immune responses such as the induction of delayed- type hypersensitivity by way of macrophage activation, and the activation of cytotoxic T-cells. Th cells belonging to the Th2 subset ("Th2 cells") secrete IL-4, IL-5, IL-6, and IL-10. Th2 cells produce cytokines which favour a humoral immune response and function as effective helper cells in B-cell activation. Thus, the selective activation of Th1 or Th2 cell types may control the type of immune response which occurs.

In this application, the terms "Th2-like" and "Th2-type" cells refer to cells having a cytokine production profile similar to that typical of Th2 cells. However Th2 type cells and Th2-like cells as defined in this application need not necessarily be conventional Th2 cells. In this application, the terms "Th1-like" and "Th1-type" cells refer to cells having a cytokine production profile similar to that typical of Th1 cells. However, Th1-type cells and Th1-like cells as defined in this application need not necessarily be conventional Th1 cells.

The importance of the subtype of Th cell which is activated is increased by cross- regulation between Th1 and Th2 subtypes. The secretion of IFN-γ by Th1 cells inhibits the proliferation of Th2 cells, and the secretion of IL-10 by Th2 cells results in decreased activation and cytokine secretion by Th1 cells. Not only do the cytokines secreted by each Th cell subtype result in decreased activity by the other Th cell subtype, but in some cases the cytokines secreted by one Th cell subtype act to down regulate immune responses of the variety stimulated by the other Th cell subtype through pathways separate from the other Th cell type. For example, the secretion of IFNγ and IL-2 by Th1 cells inhibits the production of IgE which is stimulated by IL-4 produced by Th2 cells.

The manifestation and clinical outcome of some disorders appears to depend at least in part on the balance of cytokine production typically associated with Th1 and Th2-type immune responses. One widely studied disease in which the Th1/Th2 related cytokine response ratio is important is leprosy. Leprosy is caused by the infection of macrophages by the intracellular pathogen *Mycobacterium leprae*, which may trigger either a Th1 or a Th2-type immune response. In tuberculoid leprosy a Th1-type response occurs and the patient's body mounts a cell-mediated immune response which is usually effective in slowing disease progression and allowing the patient to survive. In contrast, in lepromatous leprosy a Th2-type response suppresses the cell-mediated immune response and a humoral response arises instead. This leads to a massive expansion of the pathogen load in the patient's tissues causing severe tissue damage and greatly diminished chances for survival.

Recent experiments have suggested that a shift from a Th1 dominant cytokine profile to a Th2 dominant cytokine profile correlates with disease progression in patients suffering from HIV infection. Th2 cells secrete IL-3, IL-4, IL-5 and IL-10.

Th1 : Th2 -like cytokine ratios also appear important to Type 1 hypersensitivity reactions. Type 1 hypersensitivity reactions include common allergic reactions such as hay fever and hives, as well as disorders such as asthma and systemic anaphylaxis. The mechanism for type 1 hypersensitivity reactions involves the antigen-induced crosslinking of IgE bound to mast cells and basophils, causing the release of vasoactive mediators. Cells having a Th1-like cytokine profile reduce type 1 hypersensitivity responses, whereas cells having a Th2-like cytokine profile tend to enhance them. The Th2-type secretions IL-3, IL-4, IL-5 and IL-10 affect B-cells, mast cells, and eosinophils to stimulate the reaction. In contrast, cells having a Th1-like cytokine profile secrete IFNγ which decreases IgE production, inhibits the proliferation of Th2 cells and inhibits the type 1 hypersensitivity response. Thus, the ratio of cells having Th1 and Th2-type cytokine profiles likely influences the balance of IFNγ and IL-4, and can potentially be used to control the onset and severity of type 1 hypersensitivity reactions.

There is evidence in the art indicating a role for the ratio of cells having Th1 and Th2 type cytokine profiles in the development and progression of autoimmune disease. The majority of organ-specific autoimmune diseases develop as a consequence of the activity of $CD4^+$ T-cells which react to self antigens. Experiments which have examined the Th1/Th2-type cytokine ratio in patients susceptible to or suffering from autoimmune disease have shown a tendency for high levels of Th1-type cells to promote autoimmune responses, whereas high levels of Th2-type cells tend to protect against disease development and slow the progression of manifested autoimmune disease.

Autoimmune diseases represent a major health problem in western countries in terms of both personal suffering and health care spending. Major T-cell mediated autoimmune disorders include insulin-dependent diabetes mellitus (IDDM),

and multiple sclerosis (MS).

IDDM is an organ-specific autoimmune disorder in which insulin-producing cells of the pancreas are destroyed as a result of the infiltration of the pancreas by large numbers of self-reactive T-cells which mediate delayed-type hypersensitivity. Experiments designed to correlate the relative propensity of individuals to mount Th1 or Th2 immune responses against glutamic acid decarboxylase, a known self antigen in IDDM, with IDDM susceptibility indicate that individuals prone to mount a Th1-type cell mediated immune response are more likely to develop IDDM and tend to show more rapid disease progression than those who mount a Th2-type humoral immune response.

An animal model for IDDM has been developed using a mouse strain called nonobese diabetic (NOD) mice. NOD mice spontaneously develop insulin dependant diabetes following the infiltration of the pancreas by large numbers of self-reactive T-cells.

A common feature of human IDDM patients and NOD mice is T-cell proliferative unresponsiveness. It has been proposed that this thymic T-cell anergy may lead to the breakdown of self-tolerance and the development of autoimmunity in NOD mice. Thymic T-cell anergy in NOD mice has been linked to defective signal transduction in response to TcR binding.

Thymic unresponsiveness in NOD mouse thymocytes has been reversed *in vitro* through the addition of exogenous IL-4 to the culture medium. *In vivo*, the administration of IL-4 to prediabetic NOD mice has been shown to protect them from the onset of diabetes during the treatment period. It has been shown that *in vitro*. NOD T-cells fail to product enough IL-4 to support their own proliferation, and exogenous IL-4 completely restored the proliferative capacity of these cells *in vitro*.

T-cells, as well as other cells of the immune system, express IL-4

receptors on their cell surface. Upon binding to its receptor, IL-4 is capable of increasing the responsiveness of that cell to IL-4 by signaling an increase in IL-4 receptor expression. IL-4 promotes the development of Th2 cells from naive T-cells upon antigen stimulation and can act to further expand and stimulate these Th cells. IL-4 also inhibits macrophage activation and can inhibit most of the macrophage activating effects of IFNγ.

Experiments using NOD mice have shown that T-cells from a diabetic NOD mouse are capable of causing diabetes by adoptive transfer into an otherwise normal mouse which has been irradiated to destroy its own immune system. Moreover, a NOD mouse which has not yet become diabetic and which has been irradiated to destroy its immune system can be protected from subsequent diabetes by the adoptive transfer of normal T-cells from a healthy donor. This is consistent with a model for insulin-dependant diabetes in which self-reactive T-cells are responsible for the onset of disease.

MS is a systemic autoimmune disease which affects the central nervous system. In MS, self-reactive T-cells cause inflammatory lesions on the myelin sheath of nerve fibers, causing the destruction of the myelin which insulates the nerve fibers. The resultant loss of myelin leads to serious neurological dysfunctions.

An experimental model for MS has been developed by injecting rats with myelin basic protein. This results in a disorder called experimental autoimmune encephalomyelitis (EAE) in which self-reactive T-cells infiltrate the myelin sheaths of the central nervous system, causing the destruction of the myelin and resulting in paralysis. When self- reactive T-cells are isolated from animals suffering from EAE and injected into healthy animals by adoptive transfer, the injected animals develop EAE. However, if the isolated T-cell population is divided into Th1 and Th2 cell subtypes, only Th1 cells will cause EAE by adoptive transfer. Th2 cells do not cause EAE by adoptive transfer and can also help to protect healthy mice from developing EAE if they are subsequently injected with myelin basic protein.

The different roles played by cells having Th1 and Th2-type cytokine profiles, and their ability to suppress the activities of the Th cell subtype with the alternate cytokine secretion profile, make it highly desirable to have a means of producing enriched populations of each cell type. However, the processes regulating the differentiation of T-cell subtypes is poorly understood. The leading model proposes that the differentiation of Th cells into Th1 or Th2 subtypes is determined by the cytokine environment at the time of Th cell interaction with antigen. In particular, the interaction of a Th cell with antigen in the presence of IL-4 is believed to be necessary for Th2 cell development, and the interaction of a Th cell with antigen in the presence of IL-12 is believed to be necessary for normal Th1 cell development.

The capacity to prevent or regulate certain disorders through the manipulation of the T-cell subtypes in the patient makes it desirable to have a process for the selective enrichment of particular T-cell subtypes.

Methods for the mechanical separation of Th1 and Th2 cells from mixed cell populations *in vitro* are known in the art. However, these methods require large initial cell populations in order to allow the recovery of useful quantities of Th1 or Th2 cells. Moreover, mechanical separation can prove stressful to cells and may reduce the usefulness of the recovered cells.

It is therefore desirable to have a process which will allow the production of large T-cell subpopulations with a desired cytokine profile from a range of starting materials without the need for mechanical separation. Additionally, thymic stromal cells which are normally present during T-cell development are a potential source of secreted factors which can complicate the regulation of culture conditions and which, if derived from a non-autologous source, may introduce pathogens into the culture. Thus, it is desirable to have a process which allows the use of a culture medium in which T-cells can proliferate and differentiate in the absence of thymic stromal cells.

SUMMARY OF THE INVENTION

It is thus an object of the present invention to provide a process for the in vitro expansion and proliferation of T-cells.

It is a further object of the present invention to provide a process for the proliferation and differentiation of immature T-cells (thymocytes) to single positive T-cells, with a preponderance of selected sub-populations of T-cells.

It is a further a more specific object of the present invention to provide a process for preparing enriched populations of Th2-like lymphocytes, useful in cell therapy to alleviate symptoms of an immunological disease.

The present invention utilizes a specific type of cell suspension conditioned culture medium CM, to promote the proliferation and differentiation of particular T-cell subtypes in the absence of thymic epithelial cells. As the specific examples demonstrate, primary murine thymocytes cultured in this CM-based medium expand about 5-20 fold during 4-6 days of culture, depending on the strain and age of the mice, and may be expanded still further by 10^2-10^3 fold upon serial passage of the thymocytes. In view of the very low concentration of IL-4 ($\leq$ 0.02 ng/ml) in CM, the use of CM allowed demonstration of the fact that IL-4 plays an important role in the regulation of thymocyte differentiation, and led to the finding that IL-4 augments the differentiation of $CD4^+CD8^-$ SP thymocytes and $CD4^-CD8^-$ $TcR\alpha\beta^+$ DN thymocytes, and that this differentiation correlates with the up-regulation of surface CD69 expression on $CD4^+CD8^+$ DP thymocytes. Thus, the disclosed process of culture in CM provides a means to selectively expand T-cell subtypes *in vitro*. From immature, double positive (DP) T-cells (thymocytes) CD4+CD8+, there can be selectively differentiated *in vitro* single positive lymphocytes, to provide a cell culture containing an enriched population thereof. Such cells can, in accordance with the present invention, be cultured *in vitro* to provide an enriched population of IL-4 producing cells useful in cell therapy to combat autoimmune disease such as diabetes mellitus.

Operating the process of the invention accordingly allows the production from a starting cell population exhibiting no IL-4 secreting cytokine profile, of a cell

population having significant, even predominant, Th2-type cytokine secretory profile, which includes IL-4 secretion, IL-3 secretion and IL-10 secretion. The process thus provides a means of obtaining cell populations useful in administration to patients having certain disorders, and a process of alleviating the symptoms of such disorders by administering to patients cell populations cultured according to processes described herein.

The present invention thus provides a mammalian T-cell population which is enriched in cells having a Th2-like cytokine production profile, e.g. IL-4 production, as compared with a similar T-cell population which has not been cultured using CM or XLCM™ as described in the culturing process herein. Such an enriched T-cell population provides cells for administration to patients to alleviate T-cell mediated disorders where Th1 : Th2 ratios are believed to be important, and disorders where IL-4 is effective. The enriched T-cell population of the invention may be produced by other methods besides culturing in the presence of CM or XLCM™ as described herein, for example by culturing in media containing only the essential ingredients of XLCM™ or ingredients functionally equivalent thereto, or by totally synthetic media. The T-cell population enriched in cells having a Th2-like cytokine production profile, useful as a source of cells for treating disorders as discussed above, constitute a preferred aspect of the present invention, independently of the method by which such populations are obtained. One skilled in the art can determine whether such an enriched cell population has been obtained by conducting simple routine experiments with his starting cell population and a CM described herein, and comparing the cytokine profile of the starting cell population with that resulting from culturing in the presence of CM as described.

The present invention thus provides a mammalian T-cell population which is capable of suppressing self-reactivity to specific self antigens by self-reactive T-cells, as compared with a similar T-cell population which has not been cultured using CM or XLCM™ as described in the culturing process herein. Such a self-reactivity suppressing T-cell population provides cells for administration to patients to alleviate

the symptoms of disorders where a T-cell mediated autoimmune response is believed to contribute to disease development, progression, or symptoms. The self-reactivity suppressing T-cell population of the invention may be produced by other methods besides culturing in the presence of CM or XLCM™ as described herein, for example by culturing in media containing only the essential ingredients of XLCM™ or ingredients functionally equivalent thereto, or by totally synthetic media. The self reactivity suppressing T-cell population, useful as a source of cells for treating certain disorders as discussed above, constitutes a preferred aspect of the present invention, independently of the method by which such populations are obtained. One skilled in the art can determine whether a self reactivity suppressing T-cell population has been obtained in respect of a particular disorder by conducting simple routine experiments with his starting cell population and a CM described herein, and comparing the impact of the introduction of a suitable quantity of cells from such a population into a suitable subject suffering from the autoimmune disorder with the impact of the introduction of a suitable quantity of cells from a population resulting from culturing in the presence of CM as described. For example, the generation of a population with self-reactivity suppressing properties in relation to autoimmune diabetes could be assayed by comparing the incidence of diabetes in a suitable Scid or otherwise MHC compatible subject which is disposed to develop autoimmune diabetes following the introduction of cells cultured in the presence of CM or XLCM™ according to the method of the invention with the effect of the introduction of the starting cell population.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other advantages of the invention will become apparent upon reading the following detailed description and upon referring to the figures in which:-

FIGURE 1 is a graphical presentation of the results of Example 1 below;

FIGURE 2(A-H) is a set of graphical and pictorial presentations of the results of Example 2 below;

FIGURES 3A and 3B are pictorial and graphical presentations of the results of Example 3 below;

FIGURES 4A and 4B are pictorial and graphical presentations of the results of Example 4 below;

FIGURE 4C is a pictorial presentation of the results of Example 4A below;

FIGURES 5A and 5B are pictorial and graphical presentations of the results of Example 5 below;

FIGURE 7 is (A) a pictorial presentation and (B) a graphical presentation of the results of Example 7 below;

FIGURE 8 is (A) a pictorial presentation and (B) is a graphical presentation of the results of Example 8 below;

FIGURE 9 is a graphical presentation of the results of Example 9 below;

FIGURE 10 is a graphical presentation of the results of Example 10 below;

FIGURE 11 is a graphical presentation of the results of Example 11 below; and

FIGURE 12 is a pictorial presentation of the results of Example 12;

FIGURE 13 is a graphical presentation of the results of Example 13 below;

FIGURE 14 is a graphical presentation of the results of Example 14

below;

FIGURE 15 is a graphical presentation of the results of Example 15 below;

FIGURE 16 is a graphical presentation of the results of Example 16 below;

FIGURE 17 is a graphical presentation of the results of Example 17 below;

FIGURE 18 is a graphical presentation of the results of Example 18 below;

FIGURES 19A and 19B are graphical presentations, and FIGURE 19C is a pictorial presentation, of the results of Example 19 below; and

FIGURE 20 A is a pictorial presentation of the procedure of Example 20, below.

FIGURE 20 B is a graphical presentation of the results of Example 20 below.

FIGURES 21A and 21 B are graphical presentations of the results of Example 21, below.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As embodied and broadly described herein, the present invention is directed to a process for the production of cell populations rich in T-cells which are capable of secreting significant quantities of IL-4, along with other cytokines

charactoristic of a Th2-like cell, to methods for culturing such subpopulations, to such subpopulations produced by this process, and to methods for the uses of these subpopulations in cell therapy and other appropriate medical applications. The culturing methods are in vitro methods using conditioned medium CM as described below, as a constituent of the cell culture medium. Whilst it is probable that the major proportion of the Th2-like cells produced herein are $CD4^+$ single positive cells, this is not necessarily or exclusively so. Certain of them may be $CD8^+$ single positive cells, or potentially even double positive of double negative cells. The common, defining charactoristic of the cell subpopulation enriched is their cytokine production profile which is charactoristic of Th2-like cells, especially in respect of the capacity to secrete IL-4.

According to one preferred embodiment of the invention, there is provided a process for the differentiation and proliferation of T-cells of various types, including DP thymocytes, i.e. CD4+CD8+ T-cells, to produce cell populations enriched in single positive T-cells, CD4+ or CD8+ and/or sub-populations of such cells, in a pre-selected manner, which comprises culturing the DP thymocytes in a special conditioned medium CM described herein, optionally also in the presence of added amounts of supplements such as cytokines and plasma.

Another, more specific preferred embodiment of the invention provides a method whereby a starting population of cells containing a complex mixture of T-cells, for example splenic cells, can be cultured *in vitro* to provide a population which is significantly enriched in a specific subset of T-cells, such as Th1-like or Th2-like cells. This provides a cell composition useful for introduction into the system of a mammalian patient for exerting protective effects against certain types of autoimmune disease. The starting cell population for use in the present invention is one which comprises T-cells. Accordingly, substantially any source of mammalian T-cells may be used as the starting cell population.

The conditioned medium CM used in the process of the present invention comprises a mixture of cell factors having a balance of stimulatory and inhibitory effects

favouring the proliferation of the desired cell population. The CM composition is produced by treating a cell population with an inducing agent which includes at least one plant mitogen. Preferred such plant mitogens include plant lectins such as concanavalin A (ConA) or phytohemagglutinin (PHA), and T-cell mitogens such as mezerein (Mzn) or tetradecanoyl phorbol acetate (TPA). Especially preferred is a combination of ConA and Mzn. Other mitogens of non-plant origin, including interferons of various kinds, may be used in addition. The starting cell population used to prepare the CM may comprise peripheral blood cells, umbilical cord blood cells, bone marrow cells, mixtures of two or more types of such cells, or fractions or mixed fractions of such types of cells. The starting cell population may be induced by adding the inducing agent(s) to an appropriate suspension thereof in aqueous, nutrient-containing medium. The CM inducing process may be affected by factors produced by the cells during culture, and by culturing conditions such as the medium used, temperature, time of culture, pH, exogenous recombinant growth factors, nutrients, etc. The medium used may be serum free.

A specific preferred example of a CM for use in the process of the present invention is XLCM™. The medium XLCM has been disclosed previously - see, for example, Skea et al., "Large ex vivo expansion and reduced alloreactivity of umbilical cord blood T- lymphocytes", Blood 90: 3680 (1997). It is further described in detail in the Materials and Methods, below. It may be derived from the supernatant of activated blood mononuclear cells. It can expand human cord blood T cells > 10^4-fold and adult T cells > 10^5-fold during a 4 week culture period. XLCM™ consists of many cytokines, some of which are present in high concentration (e.g. GM-CSF, IL-2 and IFN-γ) while others are present in extremely low amount (e.g. IL-4 and IL-7). In the processes of the invention, the CM as exemplified by XLCM is preferably used as an added ingredient to a standard cell culture medium, serum containing or serum free, in amounts in the approximate range of from 5% to 40% of the total volume of the culture medium.

XLCM™ supports the differentiation and proliferation of DP thymocytes

into single positive T-cells, as evidenced by experiments reported herein on cells from laboratory mice of various strains. When the culture medium additionally includes added amounts of exogenous IL-4, the differentiation of single positive CD4+ cells is strongly favoured, thereby providing a convenient and relatively rapid means for producing enhanced populations of CD4+, Th cells. Thus the process of the invention may also be used to promote the differentiation of DP into $CD4^+$ SP thymocytes, by culturing in XLCM™ in the absence of thymic stromal cells, when IL-4 is added to the culture. This differentiation is accompanied by an increase in CD69 surface expression on $CD4^+$ $CD8^+$ DP thymocytes, suggesting that IL-4 may provide and/or induce the signals required for the differentiation of DP thymocytes into $CD4^+$ SP thymocytes.

Moreover, culturing of splenic T-cells in the presence of XLCM has been found, in accordance with the present invention, to lead to differentiation of IL-4 producing cells, especially when conducted in the absence of splenic accessory cells (antigen presenting cells, APCs). Thus the present invention also provides a convenient and relatively rapid means for producing cell populations enriched in cells exhibiting a Th2-type cytokine production profile, or a cell population in which the ratio of Th1 cytokine profile cells : Th2 cytokine profile cells is shifted from the normal in favour of the Th2-like population. Such a shift influences the onset of certain autoimmune diseases, namely by delaying their onset. Accordingly such cell populations show potential in cell therapy for treating or delaying the development of certain autoimmune diseases such as IDDM.

As described in the specific examples below, this was demonstrated by culturing T-cells obtained from mouse strains by the process of the invention, and injecting the resultant cultured cell populations into mice of other strains, and then challenging the injected mice with autoimmune disease, namely IDDM. T-cells from the mouse strain nonobese diabetic (NOD) mice, which spontaneously develop IDDM, have been analyzed for their capacity to differentiate and proliferate in XLCM™. The profile of cytokine production from the resulting cell population evidences a shift in cell subpopulations in favour of cells having a Th2-like cytokine profile with a relative

decrease in Th1-like cells. Introduction of splenic cells from diabetic NOD mice, cultured in XLCM™ into NOD Scid mice protected these from the onset of IDDM. This provides further evidence of a shift in subpopulation in favour of cells having a Th2-like cytokine profile, and the utility of such enriched cell populations in conferring enhanced protection against the onset or development of autoimmune disease.

Upon administration of T cells cultured in XLCM to a subject genetically predisposed to autoimmune diabetes (i.e. a NOD mouse), the induction of diabetes by diabetogenic T-cells subsequently added, or the induction of diabetes by existing T-cells in non-diabetic NOD mice, is inhibited. The strength of the protective effect observed is related to the interval between the time of administration of the XLCM cultured cells and the time of challenge by diabetogenic cells. Moreover, T-cells removed from subjects genetically predisposed to diabetes in which disease progression has been delayed or prevented by the prior administration of XLCM-treated T-cells have a protective effect which inhibits the development of diabetes by a second subject receiving those cells. Thus, XLCM cultured T-cells introduced into a subject are able to exert a regulatory effect on subsequent pathogenic activity by T-cells removed from that subject.

When $CD4^+$and $CD8^+$ T-cell types are isolated prior to culturing in the presence of XLCM, followed by adoptive transfer into NOD.Scid mice, it becomes clear that culture in the presence of XLCM™ modulates the behaviour of both $CD4^+$ and $CD8^+$cells in a manner relevant to the regulation of autoimmune diabetes. In particular, $CD4^+$ T-cells which have been cultured in XLCM™ are less diabetogenic than uncultured cells, and the addition of IL-4 to the culture medium further reduces their diabetogenicity. $CD8^+$ T-cells cultured in XLCM do not appear to cause diabetes by adoptive transfer, and can act to protect against the development of diabetes resulting from challenge with uncultured diabetogenic cells.

The invention accordingly provides processes for generating cell populations for administration to patients to alleviate autoimmune diseases of the T-cell

mediated type, as exemplified by IDDM. Culturing of immature T-cells (thymocytes) in the presence of the medium described herein alters the cytokine secretion profile of cells within that population, generating an increased number of IL-4 producing cells. These IL-4 producing cells can be used as drug delivery means, and administered to patients to undergo *in vivo* secretion of cytokines such as IL-4 at locations where the IL-4 is most needed. This avoids many of the side effects of systemic cytokine administration.

In a patient suffering from IDDM, the defective immune system of the patient has effectively damaged the pancreatic islet cells so that adequate insulin quantities are no longer produced. Administration of fresh, effective islets to such patients e.g. by cell therapy in generally ineffective, since the immune system attacks the newly administered islet cells. The present invention provides a cell population which can be administered along with islet cells to a diabetic patient, to counteract the tendency of the patient's immune system to attack the islet cells, and without the need to provide special protection e.g. encapsulation of the islet cells.

The invention is exemplified and demonstrated in the following specific experimental examples.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Female NOD/Del (NOD) mice, MHC class I and class I deficient DKO NOD mice, NOD.Scid mice, and NOR mice were bred and maintained in our (University of Western Ontario, Robarts Institute, London, Ontario, Canada) specific pathogen free animal facility, and were used at 6-10 weeks of age.

Thymocyte isolation

Single cell thymocyte suspensions were prepared according to standard procedures. $CD4^+CD8^+$ DP thymocytes were purified by panning on anti-CD8 mAb coated culture dishes resulting in $\geq$ 95% $CD4^+CD8^+$ thymocytes and $\leq$ 1% $CD8^+$ SP thymocytes as assayed by two-color flow cytometry.

Medium, cytokines and antibodies

XLCM™ was prepared from human cord blood as described by Skea et al., op. cit. HBCM-2 medium consists of AIM-V serum-free medium (Life Technologies, Grand Island, NY) supplemented with 20 units/ml heparin (Organon Teknika, Inc, Toronto, ON), 50 μM 2-mercaptoethanol (2-ME, Life Technologies), 10 μg/ml gentamycin sulfate and 50 μg/ml streptomycin (Sigma, St. Louis, MO). RPMI-1640 (R5F) or DMEM (D10F) (both from Life Technologies) were supplemented with 5 or 10% fetal calf serum (FCS, Life Technologies), respectively, 2 mM glutamine, 50 μM 2-ME, 100 U/ml penicillin and 100 μg/ml streptomycin. EL4.IL-2 supernatant was prepared by stimulation of EL4.IL-2 thymoma cells (10^6/ml) (American Tissue Culture Collection (ATCC), Rockville, MD) in D10F, with 20 ng/ml PMA (Sigma) for 24 h. Recombinant IL-4 was purchased from R & D Systems (Minneapolis, MN). Rat anti-mouse CD8 (TIB- 210, clone 2.43, IgG2b) and rat anti-mouse IL-4 (HB-188, clone 11B11, IgG1) mAbs were generated from ATCC cell lines and purified from culture supernatants by protein G affinity chromatography.

Cell culture

Unfractionated or purified $CD4^+CD8^+$ DP thymocytes were cultured for 4–6 d at 37°C in HBCM-2 medium supplemented with 5% XLCM™ and with or without 2.5% human cord plasma (CP). CP was prepared according to standard procedures. Thymocytes were plated (2 x 10^5/well, 2 ml/well) in 24-well tissue culture plates (Nunc, Nalge Co. Rochester, NY). To monitor thymocyte expansion in XLCM™,

cells were harvested and subcultured at the indicated time points. Control cultures contained thymocytes maintained in either R5F supplemented with XLCM™ (5 or 10%) or EL4.IL-2 supernatant (25%). Where indicated, empirically determined optimal concentrations of IL-4 (5 ng/ml) or IL-4 plus the 11B11 anti-IL-4 mAb (10 μg/ml) were added to cultures.

Intracellular cytokine expression

Thymocytes cultured in XLCM™ were fluorescently stained for the cell surface markers CD3, CD4, and CD8 or the intracellular cytokines IL-2, IL-4, IL-10 and IFN-γ, respectively. For three-color surface marker staining, thymocytes were harvested on day 4-6 of culture depending on the cell density, washed in PBS/1% BSA/0.1% sodium azide, and were stained at 4°C for 30 min with PE-conjugated anti-mouse CD4 (clone: CT-CD4, rat IgG2a; Cedarlane, Hornby, ON, Canada), PE-Cy5-conjugated anti-mouse CD8a (clone: CT-CD8, rat IgG2a; Cedarlane) and FITC-conjugated anti-mouse CD3e (clone: 500-A2, hamster IgG; Cedarlane) or FITC-conjugated anti-mouse CD69 (clone: H1.2F3, Hamster IgG, PharMingen, San Diego, CA). For three-color intracellular cytokine staining, thymocytes were initially stained with FITC-conjugated anti-mouse CD4 (clone: RM4-5, rat IgG2a; PharMingen) and PE-Cy5-conjugated anti-mouse CD8a. After two washes, the cells were fixed with Cytofix/Cytoperm™ solution, followed by two washes with Perm/wash™ solution as instructed by the manufacturer (PharMingen). Cells were then stained for 30 min at 4°C with PE-conjugated rat anti- mouse mAbs to IL-2 (clone: JES6-5H4, IgG2b), IL-4 (clone: 11B11, IgG2b), IL-10 (clone: JES5-16E3, IgG2b) or IFN-γ (clone: XMG1.2, IgG1) (all mAbs supplied by PharMingen), respectively, washed twice with PBS and fixed in 0.5% paraformaldehyde in PBS prior to flow cytometric analysis. FITC-, PE-, and PE-Cy5- conjugated isotype mAbs were used as controls. Ten thousand events were collected on a FACScan cytometer and analyzed using CellQuest™ software (Becton Dickinson, Mountain View, CA).

Diabetes monitoring

Diabetes was monitored in mice by testing urine glucose twice a week. A mouse was classified as diabetic when it urine glucose level exceeded 56 mmol/L.

Analysis of XLCM

As shown in Table I, XLCM™ was determined by ELISA to contain various concentrations of several cytokines, including IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-12, IL-13, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). XLCM™ also contains the macrophage inflammatory protein-1α (MIP-1α) and MIP-1β C-C chemokines. IL-4 (≤ 0.02 ng/ml), IL-7 (≤ 0.001 ng/ml) and IL-15 (≤ 0.008 ng/ml) are present in relatively low concentrations, and do not increase relative to normal plasma (Table I). Some XLCM™ cytokines, e.g. IL-1β, TNF-α, TGF-β, and GM-CSF, may be derived from thymic stromal cells that play an important role in thymocyte differentiation and proliferation. This is expected as XLCM™ is derived from the supernatants of mitogen-activated human cord blood nucleated cells and cord blood is an enriched source of hematopoietic stem cells which may produce various cytokines upon activation. Thus, XLCM™ is enriched with thymic stromal cell derived factors and may influence thymocyte differentiation and proliferation.

Adoptive Transfer of Cells into NOD.Scid Mice

Splenic T-cells were isolated from a diabetic NOD mouseusing a T-cell Enrichment Column (R&D Systems) and cultured in HBCM-2 containing 5 % XLCM™ for 4 - 7 days under standard tissue culture conditions (initial plating density 200 000 cells per well in 24 well plates). The resultant cells (referred to as X-DT cells) were harvested and injected intraperitoneally (i.p.) (5 000 000 cells per mouse) into female NOD.Scid mice (6 - 8 weeks of age). In certain experiments, mice were also injected i.p. with uncultured diabetogenic cells from diabetic NOD mice (DT cells) (5 000 000 cells per mouse).

TABLE 1 - CYTOKINES AND CHEMOKINES IN XLCM

Cytokine	Concentration* in XLCM™ (ng/ml)	Range (ng/ml)	Increase Relative to Plasma**
IL-8	234	181-> 1000	5200
TNF-β	112	98-160	7000
MIP-1α	98	68-243	44
IL-2	44	12-159	244
TGF-β1	21	6.9-44	***
RANTES	15	4-54	1.5
MIP-1β	11	1-39	3.4
GM-CSF	11	0.7-24	11000
TNF-RII	9.1	6.8-17	1.1
IL-1β	6.4	0.2-18	2133
M-CSF	5.4	2.3-9.7	4.5
IL-13	3.6	1.5-13	300
IFN-γ	3.6	0.6-14	89
IL-1α	2.3	0.004-4.9	2300
IL-16	2.1	0.5-6	23
TNF-RI	1.8	1.1-2.4	***
Fas	1.3	<0.04-2.3	***
TNF-α	0.37	<0.001-3.4	370
IL-12	0.26	0.07-0.8	***
SCF	0.2	0.15-0.29	***
IL-10	0.02	0.007-0.2	5.7
IL-6	0.007	<0.006-0.028	***
IL-4	0.0068	0.00012-0.08	***
IL-7	<0.001	<0.001-0.024	***
IL-15	<0.008	<0.008	***

* median concentration of cytokine measured in n=6-18 independent lots of XLCM™ using commercial ELISA kits: IL-1β, IL-2, IL-4, IL-10, IL-12, RANTES, TNF-RI, TNF-RII, Fas (Cytoscreen™, Biosource International, Camarillo, CA), INF-γ (DuoSet™, Genzyme Diagnostics, Cambridge, MA), TNF-α, GM-CSF, MIP-1α (Cytokine Direct™, Intergen Company, Purchase, NY) and MIP-1β, TNF-β, IL-1α, SCF (Quantikineα™, R & D Systems, Minneapolis, MN)

** median concentration in XLCM/median concentration in plasma

*** not increased relative to plasma level

EXAMPLE 1 - XLCM supports thymocyte proliferation

The capacity of mouse thymocytes to proliferate in XLCM (5% XLCM in serum-free HBCM-2 medium) was determined. The results are shown in Figure 1, plots of numbers of cells per well against time of culture, in days. To obtain the upper plots *A*, C57BL/6 and NOD thymocytes were cultured in 5% XLCM in HBCM-2 medium in the presence or absence of 2.5% CP. To obtain the lower plots *B*, BALB/c thymocytes were cultured in 5% XLCM in HBCM-2 medium or R5F supplemented with either 5-10% XLCM or 25% EL4.IL-2 supernatant, harvested at day 4 and then serially passaged at the indicated times. All cultures were established at a cell density of 2 x 10^5/well in 2 ml in 24-well plates. The number of cells were counted in triplicate and expressed as the number of cells per well. The variation in numbers of cell/well between wells was less than 10% at each time point. Representative data from one of three reproducible experiments are shown.

Whereas NOD and BALB/c thymocytes plated at 2 x 10^5 cells/well expanded about 5-20 fold during culture for 4-6 days in XLCM, C57BL/6 thymocytes proliferated poorly (Fig. 1). Upon serial passage, thymocytes expanded by about 10^2- to 10^3-fold during 2-4 weeks in culture, and varied according to the strain (Fig. 1) and age of the mice (data not shown). NOD and C57BL/6 thymocyte proliferation was enhanced in the presence of added CP, especially during the initial 4 days of culture (Fig. 1A). IL-2 does not appear to be a major proliferative stimulus of thymocytes grown in XLCM, as BALB/c thymocytes proliferated poorly in R5F containing 25% EL4.IL-2 supernatant (Fig. 1B). Moreover, thymocytes proliferated more vigorously in HBCM-2 medium containing 5% XLCM than in R5F medium supplemented with 5-10% XLCM (Fig. 1B). These results indicate that XLCM is particularly supportive of thymocyte proliferation *in vitro*, and this level of proliferation is significantly enhanced by the addition of CP to the medium.

EXAMPLE 2 - XLCM promotes thymocyte differentiation

Proliferation of C57Bl/6 and NOD Thymocytes

The ability of thymocytes to differentiate during *in vitro* culture in XLCM was examined. C57BL/6 and NOD thymocytes (2 x 10^5/well) were cultured in 5% XLCM in HBCM-2 medium in the presence or absence of 2.5% CP, and were passaged at the indicated times shown on Fig 2A, which presents the results in graphical form, percentage of various cell types in the culture against time. At each time point, thymocytes were analyzed for the surface expression of CD3, CD4 and CD8 by three-color flow cytometry, and the percentages of $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$ thymocytes were determined. Data shown are those for $CD3^{hi}$ thymocytes and represent one of three reproducible experiments.

In the presence of CP, more than 95% of C57BL/6 and NOD thymocytes were $CD3^+$ at days 4 and 7 of culture in XLCM(Fig. 2A). However, the patterns of differentiation in XLCM differed between C57BL/6 and NOD thymocytes, particularly after the first passage (day 4) of culture. C57BL/6 thymocytes consisted predominantly of $CD4^-CD8^+$ SP cells regardless of passage. In contrast, comparable numbers of $CD4^-CD8^+$ and $CD4^+CD8^-$ NOD SP thymocytes were present in the initial cultures, whereas NOD $CD4^-CD8^+$ SP thymocytes were predominant after one passage (day 7). The proportion of C57BL/6 and NOD $CD4^+CD8^+$ DP thymocytes was reduced from 75-80% at day 0 to 15-20% at day 4 and 7 of culture in XLCM, suggesting that many DP thymocytes may have differentiated into SP thymocytes. Interestingly, the yield of NOD $CD4^+CD8^-$ SP thymocytes obtained in XLCM in the absence of CP (Fig. 2B) was higher than that in cultures supplemented with CP (Fig. 2A) and remained stable for several passages, suggesting that CP favors the differentiation of $CD4^-CD8^+$ SP thymocytes. Furthermore, in the absence of CP, the percentages of $CD4^+CD8^+$ DP and $CD4^-CD8^+$ SP thymocytes decreased in culture, while that of $CD4^-CD8^-$ thymocytes increased significantly (Fig. 2B). Despite the poor growth and low recovery of C57BL/6 thymocytes in the absence of CP, the thymocytes recovered consisted of a similar distribution of DP and SP subsets to that observed for C57BL/6 thymocytes cultured in the presence of CP (data not shown). Thus, XLCM promotes thymocyte differentiation, and the addition of CP preferentially stimulates the differentiation of $CD4^-CD8^+$ SP thymocytes.

Proliferation of MHC DKO NOD Thymocytes

In order to demonstrate that this effect did not derive from the proliferation of SP thymocytes possibly present in small numbers in the starting NOD and C57Bl/6 thymocyte populations, similar experiments were conducted using thymocytes from MHC class I and class II deficient DKO NOD mice. Such mice are devoid of SP thymocytes. This enabled analysis of the growth potential of DP thymocytes in the absence of any outgrowth of SP thymocytes. MHC DKO DP thymocytes were plated (3 x 10^6 cells/well in 2 ml of 5% XLCM™) in serum-free HBCM-2 medium, and CP was either used alone or was added to XLCM™ in control cultures. The number of viable cells recovered from the cultures of XLCM™, XLCM™ plus CP, CP or medium alone at various time points is shown in Figure 2C. During the first day of culture, the number of cells decreased about 95% in XLCM™, 75% in XLCM plus CP, 55% in CP and 65% in medium alone. The number of cells recovered in the cultures of CP or medium alone progressively declined over time and no viable cells were recovered on days 3 and 5, respectively. In contrast, the number of cells recovered in XLCM™ or XLCM™ plus CP remained the same on day 2, and actually increased progressively during the next 3 days of culture. The number of cells recovered in XLCM™ on day 5 was increased about 10-fold compared to that recovered on day 1. The DP thymocyte proliferative response obtained in XLCM™ plus CP paralleled that of XLCM™, but the numbers of viable cells recovered in XLCM™ plus CP cultures exceeded that detected in XLCM™ cultures at all time points (Fig. 2C).

Analyses of the cell size showed that >80% of the cells recovered in XLCM™ after 1 day of culture were large blasts as determined by flow cytometry (FSC) (Fig. 2F). At this point, however, both small and large cells that formed cell aggregates were observed in XLCM™ plus CP cultures, with the percentage (14.3%) of large cells being less than that of small cells. Note that the percentage of large blasts obtained in XLCM™ plus CP was also considerably less than that obtained in XLCM™ after day 1 (Fig. 2F) but on day 3 of the culture the cell recoveries from XLCM™ and XLCM™ plus CP were comparable (Fig. 2C) inset). Interestingly, the switch in yield from small

to large cells in XLCM™ plus CP occurred on day 4 of culture, as small cells were not observed at this time (data not shown). Predominantly small cells were recovered from cultures in CP or medium alone throughout the culture period, and these cells were essentially dead by day 5 of culture (Fig. 2C).

Cell cycle analyses consistently showed that during the first day of culture, cells in S-phase of the cell cycle comprised about 30% in XLCM and about 8% in XLCM™ plus CP (Fig. 2D). The percentages of cells in S-phase increased over time and reached a plateau between days 2 and 3 of culture in XLCM™ or XLCM™ plus CP (Fig. 2D). In contrast, cells cultured in CP or medium alone did not enter S-phase of the cell cycle (data not shown).

To further determine the potency of XLCM™ in potentiating thymocyte growth, the dose-dependency of XLCM™ on thymocyte proliferation was examined. Fig. 2E shows that MHC DKO thymocyte proliferation as measured by the uptake of tritiated thymidine in XLCM™ is dose-dependent. The presence of CP alone in culture did not promote thymocyte proliferation, but CP significantly enhanced thymocyte proliferation when added to XLCM™ . Taken together, these findings indicate that XLCM™ can selectively induce both the death and growth of DP thymocytes. CP alone has no effect on thymocyte proliferation, but CP partially inhibits cell death and moreover enhances thymocyte proliferation when present in XLCM™ .

Differentiation of MHC DKO NOD Thymocytes

The ability of DP thymocytes from MHC DKO NOD mice to differentiate into SP cells during *in vitro* culture in XLCM™ was examined. Since DKO mice are devoid of SP thymocytes analyses of DKO DP thymocytes enabled investigation of whether SP thymocytes that grow in XLCM™ arise from the differentiation of DP thymocytes rather than from the selective outgrowth of SP T-cells that may contaminate a population of wild-type NOD DP thymocytes.

MHC DKO DP thymocytes were cultured in 5% XLCM™, harvested at various times and the surface antigen phenotype of viable cells was analyzed by flow cytometry. XLCM™ stimulated $CD4^+CD8^+$ DP thymocytes to differentiate into $CD4^+CD8^-$ (8.3%) and $CD4^-CD8^+$ (12.3%) SP thymocytes as well as $CD4^-CD8^-$ DN (41.8%) thymocytes during the first day of culture (Fig. 2G). The percentage of DP thymocytes recovered at this time was significantly reduced from 98% (before culture) to 38%. After 3 and 5 days of culture, the percentages of DP thymocytes were further decreased and this was accompanied by increased percentages of SP and DN thymocytes (Fig. 2H). This differentiation of DP to SP and DN thymocytes was delayed in XLCM™ + CP, (Figs. 2G and 2H). This is consistent with our findings that the numbers of cells in S-phase (Fig. 2D) and large cells (Fig. 2F) were low in XLCM™ + CP at this time. Subsequently, the percentage of DP thymocytes was reduced, and that of SP and DN cells increased slowly during a 3 day culture. Interestingly, the percentage of DP cells was diminished dramatically (<10%) on day 4, and this was accompanied by a switch of small to large cells, indicative of the growth and differentiation of DP thymocytes in XLCM™ + CP. The phenotypes of thymocytes on day 4 (data not shown) were similar to that on day 5 (Fig. 2H). Interestingly, DP thymocytes differentiated mainly into $CD4^-CD8^+$ SP and $CD4^-CD8^-$ DN cells in XLCM™ and XLCM™ + CP (Fig. 2H).

Example 3 - XLCM™ mediated DP thymocyte differentiation is associated with the upregulated surface expression TCR, CD25, CD44, CD69 and CD40L

During the development of $CD4^+CD8^+$+ DP thymocytes into $CD4^+$ or $CD8^+$ SP thymocytes, the surface expression of TCR as well other markers of T cell maturation (e.g. CD69, CD44, and CD25) may be upregulated. To determine whether thymocytes cultured in XLCM™ or XLCM™ plus CP are phenotypically mature, their surface expression of TCR, CD25, CD69, CD44 and CD40L was examined. MHC DKO thymocytes were cultured in XLCM™, harvested at various times, stained with fluorochrome-conjugated mAbs to CD4, CD8 and TCR$\alpha\beta$ and analyzed by flow cytometry. Expression of TCR$\alpha\beta$ on $CD4^+CD8^-$ and $CD4^-CD8^+$ SP as well as

$CD4^+CD8^+$ DP thymocytes was upregulated during the first 3 days of culture in XLCM™ (Fig. 3A). In contrast, $CD4^-CD8^-$ DN thymocytes were either $TCR\alpha\beta^{low}$ or $TCR\gamma\delta$ (data not shown), indicating that some MHC DKO thymocytes cultured in XLCM™ were able to differentiate into $TCR\gamma\delta$ thymocytes. Similar results were obtained for thymocytes cultured in XLCM™ + CP.

Before culture, MHC DKO thymocytes were $CD25^-$, $CD44^{intermediate}$ and $CD69^-$. Culture of these thymocytes in XLCM™ or XLCM™ plus CP for 5 days resulted in the elevated surface expression of CD25, CD44 and CD69, as reflected by increased percentages of $CD25^+$, $CD44^{high}$ and $CD69^+$ thymocytes (Fig. 3B). Whereas the expression of these surface markers was upregulated on all thymocyte subsets, CD69 expression was enhanced predominantly on $CD4^+CD8^+$ DP cells. CD25 expression was increased on $CD4^+CD8^-$ and $CD4^-CD8^+$ SP cells (data not shown). Culture of thymocytes in XLCM™ or XLCM™ plus CP resulted in the increased surface expression of CD40L on about 1% of these thymocytes, suggesting that the latter thymocytes acquired a mature and activated T cell phenotype (Fig. 3B).

Example 4 - Thymocytes differentiated in XLCM™ produce cytokines and respond to syngeneic and allogeneic MHC alloantigens

To examine whether MHC DKO thymocytes differentiated in XLCM™ are functionally mature, the capacity to produce Th1 and Th2 like cytokines and to respond to MHC alloantigens was determined. IL-2, IL-4, IL-10 and IFN-γ producing thymocytes were enumerated by intracellular cytokine fluorescent staining. About 5-10% of MHC DKO thymocytes cultured in XLCM™ for 5 days secreted IL-2, IL-4, IL-10 and IFN-γ (Fig. 4A). The percentage of IL-4 or IL-10 producing cells was slightly higher than that of IFN-γ-producing cells. Both $CD4^+CD8^-$ and $CD4^-CD8^+$cytokine-producing SP thymocytes were detected (data not shown). It is unlikely that these cytokine-producing cells resulted from the outgrowth of pre-existing mature SP thymocytes, as MHC DKO mice are devoid of SP cells and no cytokine-producing cells were detected in freshly isolated thymocytes.

In addition to their capacity to produce cytokines, MHC DKO thymocytes that differentiated in XLCM™ responded to syngeneic NOD ($H\text{-}2^{g7}$) and allogeneic (BALB/c, $H\text{-}2^{d}$; and C57BL/6, $H\text{-}2^{b}$) MHC antigens present on irradiated stimulator splenocytes (Fig. 4B). The response of these MHC DKO thymocytes to $H\text{-}2^{b}$ alloantigens was lower than that to $H\text{-}2^{d}$ alloantigens, which may be explained in part by the deficiency of I-E molecules expressed by antigen presenting cells (APCs) from C57BL/6 ($H\text{-}2^{b}$) mice. These results indicate that thymocytes that differentiate in XLCM™ are functionally mature.

EXAMPLE 5 - IL-4 regulates DP thymocyte differentiation in XLCM in the absence of thymic epithelial cells

C57BL/6 and NOD $CD4^{+}CD8^{+}$ DP thymocytes were isolated by panning on anti-CD8 mAb coated dishes and shown by flow cytometry to be $\geq$ 95% $CD4^{+}CD8^{+}$ DP cells. Thus, the purified population of DP cells was largely devoid of thymic stromal cells. The purified DP cells were cultured (2×10^{5}/well) in 5% XLCM in HBCM-2 medium in the presence or absence of CP (2.5%) or CP plus IL-4 (5 ng/ml). Thymocytes were harvested on days 5-6 and were stained with PE-conjugated anti-mouse CD4 and PE-Cy5-conjugated anti-mouse CD8 mAbs. Numbers in quadrants represent percentages of positive cells, and the data shown are from one of two representative experiments with similar results.

C57BL/6 and NOD DP thymocytes not only proliferated well but also differentiated under these conditions (Fig. 5A). The patterns of differentiation of C57BL/6 and NOD DP thymocytes were similar to those observed for unfractionated thymocyte populations (Figure 2A), as C57BL/6 $CD4^{-}CD8^{+}$ and NOD $CD4^{+}CD8^{-}$ SP thymocytes, respectively, were found to be predominant.

The addition of exogenous IL-4 to cultures promoted the differentiation of $CD4^{+}CD8^{-}$ SP and $CD4^{-}CD8^{-}$ DN thymocytes and diminished the proportion of

$CD4^-CD8^+$ SP and $CD4^+CD8^+$ DP thymocytes. The DN thymocytes induced to differentiate by IL-4 express TcRαβ but not TcRγδ. These results indicate that DP thymocytes can differentiate into SP thymocytes in XLCM in the absence of thymic stromal cells, and that IL-4 can promote thymocyte differentiation independently of thymic stromal cells.

IL-4 also influenced the differentiation of MHC DKO DP thymocytes in the absence of thymic stromal cells. Purified MHC DKO DP thymocytes were cultured for 5 days in XLCM™ in the presence or absence of CP and/or IL-4, and then analyzed for their surface expression of CD4 and CD8. In the presence of CP, addition of IL-4 (5 ng/ml) to culture decreased the percentage of $CD4^-CD8^-$ DN (from 37.9% to 16.1%) thymocytes, and markedly increased the percentages of $CD4^+CD8^+$ DP (from 8.4% to 18.5%) and $CD4^+CD8^-$ SP (from 5% to 20.7%) thymocytes. Similar results were observed when CP was absent from the cultures. As shown in Figure 5B, the effect of Il-4 on thymocyte differentiation was completely blocked in the presence of an anti-IL-4 mAb. Other cytokines such as IL-7, IL-10, IL-12 and IL-15 did not significantly alter $CD4^-CD8^-$ DKO DP thymocyte differentiation when added to XLCM™ based culture. Thymocyte proliferation in XLCM™ was enhanced by IL-4 but not IL-7, IL-10, IL-12 or IL-15, as depicted in Figure 5C).

EXAMPLE 6 Il-4 regulates thymocyte proliferation and differentiation in XLCM™

Since XLCM™ promotes both thymocyte proliferation and differentiation, this culture medium was used to further analyze the role of a given cytokine(s) in thymocyte differentiation. XLCM™ contains very low amounts of IL-4, IL-7, IL-10, IL-12 and IL-15, which do not exceed the levels relative to normal plasma, with the exception of IL-10 (Table 1). IL-4 is involved in the regulation of thymocyte development, but its precise role is not fully understood. The question of whether exogenous IL-4 influences thymocyte differentiation in this XLCM™-based culture system was investigated.

C57BL/6 and NOD thymocytes (2 x 10^5/well) were cultured in 5% XLCM in HBCM-2 medium in the presence of 2.5% CP. IL-4 (5 ng/ml) or IL-4 plus anti-IL-4 mAb (10 mg/ml) was added to cultures. Thymocytes were harvested on day 6, stained with PE-conjugated anti-mouse CD4 and PE-Cy5-conjugated anti-CD8 mAbs, and analyzed by flow cytometry. The results are shown in Fig. 6A. The numbers in quadrants represent percentages of positive cells. Representative data from one of three reproducible experiments are shown.

In the presence of 2.5% CP, addition of IL-4 (5 ng/ml) to cultures markedly increased the frequencies of $CD4^+CD8^-$ SP and $CD4^-CD8^-$ DN thymocytes but decreased the frequencies of $CD4^-CD8^+$ SP and $CD4^+CD8^+$ DP thymocytes from both mouse strains (Fig. 6). Similar results were obtained when CP was absent from cultures. These patterns of IL-4 induced thymocyte differentiation were completely blocked by addition of anti-IL-4 mAb to cultures. Thus, IL-4 induces mouse thymocyte differentiation in XLCM in a strain-independent manner.

EXAMPLE 7 - IL-4-mediated thymocyte differentiation is associated with an increase of surface CD69 expression

Since expression of CD69 on thymocytes may correlate with negative and positive thymocyte selection, experiments were conducted to investigate whether a change in CD69 surface expression is associated with IL-4-induced thymocyte differentiation. C57BL/6 and NOD $CD4^+CD8^+$ DP thymocytes isolated by panning on anti-CD8 mAb coated dishes and shown by flow cytometry to be $\geq$ 95% $CD4^+CD8^+$ DP cells were cultured (2 x 10^5/well) in 5% XLCM in HBCM-2 medium in the presence or absence of CP (2.5%) or CP plus IL-4 (5 ng/ml). Thymocytes were harvested on days 5-6 and were stained with PE-conjugated anti-mouse CD4, PE-Cy5-conjugated anti-mouse CD8 and FITC conjugated anti CD69 mAbs.

Figure 7A shows that C57BL/6 thymocytes express a basal level of surface CD69 when cultured in XLCM in the absence of exogenous IL-4. Exposure to

IL-4 elevated the surface expression of CD69 on thymocytes as reflected by an increase in both the percentage of $CD69^+$ thymocytes and the mean fluorescence intensity (MFI). Although upregulation of CD69 expression by IL-4 was observed on $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ thymocytes, the most significant increase (~25%) in CD69 expression was observed on $CD4^+CD8^+$ DP thymocytes (Fig. 7A). The effect of IL-4 on CD69 expression was almost completely blocked when anti-IL-4 mAb was added to cultures (Fig. 7A). IL-4 treatment increased the MFI values for CD69 expression on NOD and NOR thymocytes, although the percentages of $CD69^+$ cells in these thymocyte populations did not increase significantly (Fig. 7B).

EXAMPLE 8 - XLCM-induced Th2-like cytokine production by thymocytes is enhanced by IL-4

To determine the T-cell subtypes produced by culture in XLCM, the cytokine secretion profiles of thymocytes cultured in XLCM were analyzed. The ability of exogenous IL-4 or cord plasma (CP) to enhance the expansion of particular T-cell subtypes was examined. IL-2, IL-4, IL-10 and IFN-γ producing thymocytes were enumerated by three-color fluorescent intracellular cytokine staining in conjunction with cell surface staining for $CD4^+$ and $CD8^+$ thymocyte subsets, as shown in Fig. 8A. C57BL/6 and NOD thymocytes were cultured (2×10^5/well) in 5% XLCM in HBCM-2 for 4-6 days. CP (2.5%) and/or IL-4 (5 ng/ml) were added to cultures, as indicated. Thymocytes were harvested and stained with FITC-conjugated anti-CD4 and PE-Cy5-conjugated anti-CD8 mAbs, and then intracellularly stained with PE-conjugated anti-mouse IL-2, IL-4, IL-10 or IFN-γ mAbs, respectively. Data were analyzed by three-color flow cytometry, and results are expressed as percentages of cytokine-producing cells. Data shown are from one of three representative experiments with similar results. *A*, Fluorescence profiles of intracellular expression of IL-4 by $CD4^+$ and $CD8^+$ NOD thymocytes. *B*, Histograms of intracellular cytokine expression by ungated thymocytes and gated $CD4^+CD8^-$ and $CD4^-CD8^+$ SP thymocytes.

Although C57BL/6 thymocytes proliferate weakly in the absence of CP, sufficient cells for flow cytometric analysis were obtained. C57BL/6 and NOD

thymocytes cultured in XLCM were found to secrete IL-2, IL-4, IL-10 and IFN-γ, but mainly IL-4 and IL-10 (Fig. 8B). The frequencies of IL-4 and IL-10-producing NOD thymocytes were higher than those of C57BL/6 thymocytes. Addition of exogenous IL-4 to XLCM increased the frequencies of IL-4 and IL-10-producing C57BL/6 but not NOD unfractionated thymocytes (Fig. 8, A and B). In contrast, addition of CP to cultures strongly inhibited cytokine production by both C57BL/6 and NOD thymocytes. This inhibition was partially reversed by addition of exogenous IL-4, which increased the frequencies of IL-4- and especially IL-10- producing thymocytes (Fig. 8B). These results indicate that XLCM preferentially supports the differentiation of thymocytes that secrete Th2-type cytokines, and the frequencies of the latter thymocytes can be enhanced in culture by the addition of IL-4 and inhibited by the addition of CP.

EXAMPLE 9 XLCM Stimulates Splenic T-cell Proliferation

The experiments reported in Examples 2 and 5 considered the effect of culture in XLCM on thymocytes. However, thymocytes represent an unusual T-cell population because of the high percentage of immature T-cells as compared to other regions of the body. As clinical treatments generally rely on more easily accessible sources of T-cells, such as peripheral blood, it was desirable to assess the effect of XLCM culture on a more mature T-cell population. Peripheral blood is not a practical source of T-cells from mice. Instead, secondary lymphoid organs, and in particular the spleen, was selected as a source of predominantly mature T-cells for the examination of cell proliferation and phenotype following culture in XLCM.

Splenocytes from C57Bl/6 and NOD mice were cultured in HBCM-2 medium containing 5 % XLCM with or without cord plasma (CP) and survival was assessed after 4, 7, and 11 days in culture. The results of this experiment are depicted in Figure 9A.

CP enhanced the survival of C57Bl/6 T-cells during the first 7 days of culture, but survival at 11 days was higher in the group which were cultured in the absence of CP. NOD mouse T-cells cultured in the presence of CP had a somewhat lower survival rate than those cultured without CP until day 11 of culture at which point

most of the cells in both groups had died.

Splenocytes from BALB/c mice were cultured in HBCM-2 medium containing 5 % XLCM or R5F medium with or without 5 % or 10 % XLCM at a plating density of 200 000 cells / well in 24-well plates. The cells were harvested, counted, and passaged (at 200 000 cells / well) at days 4, 7, 11, 18 and 24. (Figure 9 B)

BALB/c T-cells cultured in the absence of XLCM died within the first 7 days of culture, with fewer than 1 000 000 cells /1 well surviving to passage at day 4. In contrast, T-cells cultured in R5F with the addition of 5 % XLCM had in excess of 3 000 000 survival as of day 4 of culture and had some viable cells in culture as of day 11. T-cells cultured in the presence of 10 % XLCM had approximately 2 500 000 surviving cells / well as of day 4 of culture, and also had some viable cells as of day 11 of culture. T-cells cultured in HBCM-2 plus 5 % XLCM had over 3 500 000 surviving cells /well on day 4. This population declined to just over 1 000 000 cells / well on day 11 and began increasing after day 18, reaching a population in excess of 2 500 000 cells / well on day 24.

Thus, XLCM can prolong the survival of T-cells from spleen *in vitro*.

EXAMPLE 10 PHENOTYPES OF SPLENOCYTES EXPANDED IN XLCM

Splenocytes from C57Bl/6 and NOD mice were cultured in HBCM-2 medium containing 5 % XLCM in the absence (Figure 10 A) or presence (Figure 10 B) of CP in 24-well plates. The cells were harvested and sequentially passaged, and their phenotype was determined using flow cytometry. T-cells cultured in XLCM in the absence of CP are predominately single positive cells.

In splenocytes from C57Bl/6 mice, the addition of cord plasma appears to disfavour the expression of $CD4^+$, and favour the expression of $CD8^+$ early in culture. The addition of CP correlated with a decrease in $CD4^-$ $CD8^-$ DN cells in later culture.

Substantially the same impact of CP was observed in splenocytes from NOD mice. However, in splenocytes from NOD mice there was also a much more pronounced impact of CP on the percentage of $CD3^+$ cells than there was with C57Bl/6 splenocytes. In splenocytes from NOD mice, the addition of CP caused an increase in $CD3^+$ cells from approximately 60 % (no CP) to in excess of 80 % in early culture and from approximately 40 % to approximately 80 % later in culture.

EXAMPLE 11 IL-4 ENHANCES THE DIFFERENTIATION OF XLCM-INDUCED IL-4 PRODUCING CELLS

Example 5 indicated that IL-4 could enhance thymocyte differentiation in XLCM. It was therefore possible that IL-4 might have some effect on spleen T cells cultured in XLCM. However, as spleen T-cells are predominantly SP cells, it was unclear what effect, if any, IL-4 would have.

Splenic T-cells from C57Bl/6 or NOD mice were cultured in HBCM-2 medium containing 5 % XLCM in the presence or absence of IL-4 (5 ng/ml) and/or CP for 5-6 days at which time they were harvested and their cytokine production profiles were measured. Figure 11 depicts the result obtained for (A) total T-cells, (B) $CD4^+$ cells, and (C) $CD8^+$ cells.

Cultures of total C57Bl/6-derived splenic T-cells exposed to XLCM with no further additions contained more IL-4 and IL-10 producing cells than IFNγ and IL-2 producing cells; however, IL-4 producing cells formed only approximately 10 % of the cell population. However, when exogenous IL-4 was added to the culture medium, IL-4 producing cells increased to nearly 20 % of the cell population. The addition of CP in conjunction with IL-4 reduced the overall percentage of IL-4 producing cells in culture to lower levels than those observed with XLCM alone. The addition of CP without IL-4 reduced the production of all measured cytokines to levels below those observed with XLCM alone. When C57Bl/6 splenic T-cell cytokine production was examined for $CD4^+$ and $CD8^+$ cells separately, a similar effect of IL-4 production was

observed. However, it also became apparent that the addition of IL-4 depresses the production of IL-2 by both $CD4^+$ and $CD8^+$ cells.

In parallel to C57Bl/6 cultures, cultures of total NOD-derived splenic T-cells exposed to XLCM with no further additions contained more IL-4 and IL-10 producing cells than IFNγ and IL-2 producing cells; however, as with the C57Bl/6 culture, IL-4 producing cells formed only a small proportion of the total cell population. IL-4 producing cells comprised only approximately 5 % of the total T-cell population. The addition of exogenous IL-4 to the culture medium resulted in a moderate increase in the percent of IL-4 producing cells, however, these cells still represented less than 10 % of the total T-cell population. In contrast to the results observed in respect of C57Bl/6 cells, the addition of both IL-4 and CP to NOD T-cell culture increased the percentage of IL-4 producing cells above the level observed for IL-4 only. The addition of IL-4 to cultures of NOD T-cells did not significantly increase the percentage of cells in the population producing IFNγ.

Thus, IL-4 enhances the differentiation of XLCM-induced IL-4 producing cells.

EXAMPLE 12 EFFECT OF XLCM ON DIABETOGENIC T-CELL DIFFERENTIATION: PROMOTION OF IL-4 PRODUCING CELL DIFFERENTIATION

Example 8 indicated that culture in XLCM caused enrichment of Th2-type cells from a starting population of thymocytes. As the Th2 : Th1 ratio may influence the behaviour of self-reactive cells and may thereby be important to the regulation of certain autoimmune diseases, including IDDM, it would be useful to have a means of predicably altering the Th1 : Th2 ratio in T-cell populations drawn from tissues other than thymus. In order to investigate the effect of XLCM culture on a more mature T-cell population than that found in thymus, the effect of XLCM culture on T-cells from spleen was examined. The NOD mouse strain was selected because a

suboptimal Th2 cell level in NOD T-cell populations has been postulated to play a role in the development of diabetes in these animals. It would, therefore, be useful to know if the predictable adjustment of the Th2 : Th1 ratio in such cell populations was possible.

A T-cell population containing self-reactive T-cells was purified from the spleens of NOD mice with active diabetes using a T Cell Enrichment Column (R&D Systems) and cultured at a plating density of 200 000 cells / well in 24-well plates. In NOD mice, self reactive T-cells are diabetogenic, because they mediate an immune response to self antigens on pancreatic cells, leading to cell loss and a corresponding loss of insulin-producing capability. If left untreated, NOD mice typically develop autoimmune diabetes at an early age. The NOD T-cells were cultured in either HBCM-2 medium only in antiCD3 coated plates, or in HBCM-2 medium containing 5 % XLCM. After 48 hours the cells were harvested and the cytokine profiles of $CD4^+$ cells was assessed using flow cytometry. The results of this experiment are depicted in Figure 12. Cells cultured in XLCM express the Th2-type cytokines IL-4 and IL-10 more highly than cells grown in HBCM-2 alone in the presence of anti CD3. The level of IFNγ, a Th1-type cytokine, was not increased by culture in XLCM. Thus, culture of NOD splenocytes in XLCM selectively enhances the expression of cytokines typically associated with the Th2 phenotype.

EXAMPLE 13 T-CELLS ISOLATED FROM DIABETIC MICE AND CULTURED IN XLCM™ ARE NOT PATHOGENIC AND DELAY ADOPTIVE TRANSFER OF IDDM

In light of Example 12 and the cross-regulation of Th2 and Th1-type cytokine related activities, the question whether NOD Th2-like cells produced by the methods of Example 11 could influence the development of diabetes in pre-diabetic NOD mice was investigated.

A population of T-cells containing self-reactive T-cells was isolated from the spleens of NOD mice with active diabetes and cultured in XLCM for 6 days. The cultured cells (X-

DT) were adoptively transferred (5 000 000 cells per mouse) into NOD.SCID mice either alone or in conjunction with uncultured diabetogenic cells (DT) (5 000 000). Control mice received only DT cells (5 000 000 cells per mouse). The results of this experiment are depicted in Figure 13.

Diabetes was apparent in some control animals by 14 days following transfer, and all control animals were diabetic within 30 days of transfer. In contrast, animals which received X-DT cells remained diabetes-free for the full 58 day observation period. Those animals which received X-DT and DT cells together developed diabetes within the observation period; however, the time of onset was later than was observed in the animals which received only DT cells. The time of earliest onset was delayed by approximately10 days, and the time of latest onset was delayed by approximately 20 days, providing an average 15 day delay in the time of onset. This indicates that not only does culture in XLCM inhibit the diabetogenic characteristics of otherwise diabetogenic self-reactive cells, but these cultured cells can also undertake a protective function *in vivo* when transferred into an animal challenged by diabetogenic cells.

EXAMPLE 14 KINETICS OF PROTECTION FROM IDDM CONFERRED BY ADOPTIVE TRANSFER OF X-DT CELLS TO NOD.SCID RECIPIENT MICE

In Example 13, X-DT cells were transferred at the same time as uncultured DT cells. To investigate whether the regulatory effect of the transferred Th2 cells would persist once those cells were removed from XLCM and placed in the environment of the host animal, the effect of a delay between the transfer of X-DT cells into an animal and the challenge of that animal with DT cells was examined.

T-cells were isolated from NOD mice expressing active diabetes. These cells were cultured in XLCM for 6 days, at which point they (X-DT) were harvested and injected (5 000 000 cells / mouse) into NOD.Scid mice. Mice in which diabetes was

not detected were challenged by the further injection 5 000 000 uncultured T-cells from NOD mice expressing active diabetes (DT). Mice were challenged with DT cells at either 1 week, 6 weeks, or 13 weeks following initial injection with X-DT cells, and the incidence of detectable diabetes in these animals was monitored. The results of this experiment are depicted in Figure 14.

By week 3, diabetes was detectable in some mice of the challenged with DT cells in week 1. All these mice suffered from detectable diabetes by week 4. In contrast, animals which were not challenged with DT cells until week 6 did not suffer from detectable diabetes until week 9, and some of these mice did not suffer from detectable diabetes until week 17, fully 11 weeks after DT challenge. Thus, the protective effect of X-DT cells is enhanced in circumstances where challenge does not occur until several weeks after transfer of the X-DT cells. Mice challenged with DT cells in week 13 did not begin to express detectable diabetes until week 16, reflecting the same 3 week disease-free period observed in respect of the mice challenged in week 6. Moreover, although the period necessary for initial onset in some mice was similar for the mice challenged in week 6 and week 13, the initial rate at which the population as a whole succumbed to detectable diabetes was lower in the mice challenged in week 13 than in those challenged in week 6. In particular, 7 weeks after DT challenge, approximately 70 % of the mice challenged in week 6 suffered from detectable diabetes. In contrast, 7 weeks after DT challenge, fewer than 50 % of the mice challenged in week 13 suffered from detectable diabetes.

Thus, not only are transferred X-DT cells capable of retaining their regulatory power after prolonged exposure to the host animal, but the regulatory effect is enhanced when a moderate prechallenge period is allowed.

EXAMPLE 15 DIABETOGENIC T CELLS CULTURED IN XLCM IN THE ABSENCE OF ACCESSORY CELLS HAVE A PREVENTATIVE EFFECT AGAINST THE TRANSFER OF IDDM IN NOD.SCID MICE

Accessory cells present antigen to T-cells and may also provide co-stimulatory signals. Accessory cells are normally considered necessary to T-cell activation. The results of Example 7, as well as the shift in cytokine profile observed in XLCM culture suggested that T-cell activation was occurring in these experiments. In light of the normal role for accessory cells in T-cell activation, the effect of the co-culture of accessory cells with NOD spleen T-cells in XLCM was investigated to determine if the presence of accessory cells enhanced the effect of XLCM culture.

A) Effect of Accessory Cells on Diabetes Protection

NOD mice suffering from active diabetes were sacrificed and splenocytes were removed. A portion of these splenocytes were purified to remove accessory cells. Both splenocyte T-cell populations with (X-DT/AC) and without (X-DT) accessory cells were cultured in HBCM-2 containing 5 % XLCM for 6 days. Diabetogenic T-cells (DT) were isolated using a T-cell enrichment column (R&D Systems) on the day of adoptive transfer. T-cells were injected intraperitoneally into female NOD.Scid mice (6-8 weeks of age) (5 000 000 cells / mouse), and the mice were monitored for the onset of diabetes. The results of this experiment are depicted in Figure 15A. NOD.Scid mice not injected with cells will eventually develop diabetes because of their genetic predisposition to this disease. Injection of DT cells accelerated the onset of diabetes in these animals, while DT cells cultured in the presence of XLCM™ for 6 days (X-DT/Ac) delayed the onset of diabetes. The removal of accessory cells prior to culture (X-DT) enhanced this protective effect.

B) Effect of Accessory Cells on Cytokine Production

Splenocytes and purified T-cells were prepared from diabetic NOD mice and cultured in the presence of absence of accessory cells as described in (A), above. The splenocytes and purified T-cells were cultured in 24-well plates (200 000 cells/well) in HBCM-2 containing 5 % XLCM for five days. The cytokine profiles of these cells were measured using flow cytometry and the results are depicted in Figure 15B. These

results indicate that co-culture with non-T-cell splenocytes reduces the production of IL-4 by T-cells. (i.e. The removal of accessory cells from splenocytes prior to culture in XLCM™ increases the proportion of IL-4 producing cells in the T-cell population obtained following culture.) Thus, Th2 cytokine producing cells are reduced in the presence of non-T splenocytes.

C) Effect of Accessory Cells on the Differentiation of Diabetogenic Splenic T-cells Cultured in XLCM™

Splenocytes and purified T-cells were prepared and cultured in the presence or absence of accessory cells as described in (B), above. The differentiation of the cultured cells was assessed using flow cytometry. The results of this experiment are depicted in Figure 15 C. These results indicate that the removal of accessory cells from splenocytes prior to culture in XLCM™ results in decreased proportions of $CD4^+$ $CD8^+$ DP cells and $CD4^+$ $CD8^-$ SP cells, and an increased proportion of $CD4^-$ $CD8^+$ SP cells. The percentage of T cells recovered in the splenocyte culture is similar to that in the purified T cell culture.

EXAMPLE 16 REPOPULATION AND PROLIFERATION OF X-DT CELLS IN NOD.SCID MICE

In light of the results of Example 15, it was desirable to determine the types of T-cells which proliferated following adoptive transfer of XLCM cultured cells. Splenic T-cells were isolated from NOD.Scid mice with active diabetes and cultured for 5 - 7 days in HBCM-2 containing 5 % XLCM. These cells were then harvested and adoptively transferred into NOD.Scid mice. Mice were sacrificed at 7, 21, 35, 60, and 120 days after adoptive transfer and $CD3^+$, $CD4^+$, and $CD8^+$T-cells were quantified using three-colour flow cytometry. The results of this experiment are depicted in Figure 16. These results indicate that $CD4^+$ T-cells are preferentially expanded *in vivo* in the period following adoptive transfer of T-cells cultured in XLCM. The results demonstrate that X-DT cells have the ability to repopulate and proliferate in NOD.Scid

mice.

EXAMPLE 17 CYTOKINE PRODUCTION BY SPLEEN T CELLS OF NOD.SCID MICE ADOPTIVELY TRANSFERRED 60 DAYS PREVIOUSLY WITH X-DT CELLS

In light of the results of Examples 12, 13, 14 and 16, it was desirable to determine the cytokine profile of T-cells in mice which had been adoptively transferred with XLCM cultured T-cells, and to relate this to the development of diabetes following challenge with diabetogenic T-cells.

T-cells from NOD mice expressing active diabetes were cultured in HBCM-2 containing 5 % XLCM for a period of 5 - 6 days. These cells (X-DT) cells were then injected into NOD.Scid mice (5 000 000 cells / mouse) either alone or in conjunction with uncultured T-cells from NOD mice suffering from active diabetes (DT cells). Sixty days later, the mice were sacrificed, and splenic T-cells were removed for cytokine production analysis by flow cytometry. The results of this experiment are depicted in Figure 17. Overall cytokine production was lower among T-cells obtained from mice that received DT cells only compared to mice that received both DT and X-DT cells. The results show that IL-4 producing cells are predominant in the spleens of NOD.Scid mice adoptively transferred with X-DT cells. A higher percentage of cytokine-producing cells was found in the large T-cell compartment. The NOD.Scid mice that were co-transferred with X-DT and DT cells and developed IDDM at day 30 to 35 had a lower percentage of cytokine-producing cells.

EXAMPLE 18 CYTOKINE PRODUCTION BY MESENTERIC LYMPH NODE T-CELLS OF IDDM-FREE NOD.SCID MICE ADOPTIVELY TRANSFERRED 60 DAYS PREVIOUSLY WITH X-DT CELLS

T-cells from NOD mice with active diabetes were cultured in HBCM-2

medium containing 5 % XLCM for 5-6 days. These (X-DT) cells were harvested and introduced into NOD.Scid mice by adoptive transfer (5 000 000 cells / mouse). Sixty days later these animals were sacrificed and mesenteric lymph node cells were removed for cytokine production analysis. The results of this experiment are depicted in Figure 18. The results indicated a significant level of IL-4 producing T-cells. In particular, the results show that IL-4 producing T-cells were predominant in the lymph nodes of IDDM free NOD.Scid mice adoptively transferred with X-DT cells, and $CD8^+$ IFN-γ-producing cells were undetectable.

EXAMPLE 19 SURVIVAL AND PHENOTYPE OF ADOPTIVELY TRANSFERRED CELLS

To investigate the relationship between the survival of transferred cells and the development of diabetes, cell populations differing slightly in the Thy 1 receptor (thereby providing a marker to distinguish the cell types), but having comparable ability to reconstitute the immune system of a Scid mouse were employed.

Two populations of T-cells from NOD mice with active diabetes were prepared. The first population (Thy 1.2) was derived from NOD mice expressing the Thy1.2 membrane marker, and these cells were cultured in HBCM-2 containing 5 % XLCM for 5-6 days. The second population (Thy 1.1) was derived from NOD mice expressing the Thy 1.1 membrane protein and was not cultured in the presence of XLCM prior to transfer.

Adoptive Transfer of Thy 1.1 and Thy 1.2 Cells

NOD Scid mice were adoptively transferred with Thy 1.2 (X-DT) T-cells (5 000 000 cells / mouse). Eight weeks later they were challenged with Thy 1.1 (DT) T-cells. The percent of mice free from detectable diabetes over the observation period is depicted in Figure 19 A. Twenty weeks after the initial Thy 1.2 transfer, the mice were sacrificed and the number of T-cells of each type from various tissues was assessed.

This data is depicted in Figure 19 B, wherein PLN refers to cells obtained from the pancreatic lymph node, MLN refers to cells obtained from the mesenteric lymph node, Figure 19 B(A) depicts the total number of T-cells of both types; Figure 19 B (B) depicts the numbers of Thy1.1$^+$ T-cells, and Figure B(C) depicts the percentage of Thy1.1$^+$ cells in lymphoid tissue. The results demonstrate that the ratio of Thy1.1$^+$ (DT) cells to Thy1.1$^-$ (X-DT) cells is higher in diabetic than non-diabetic mice.

The cytokine profile of the Thy 1.1 and Thy 1.2 cell populations obtained as described above were analyzed by flow cytometry. Splenocytes were stained intracellularly with PE-conjugated anti-mouse IL-4 or anti-mouse IFN-γ in conjunction with surface staining for CD3 with FITC-conjugated anti-mouse CD3 monoclonal antibodies, and analyzed by flow cytometry. The results of this experiment are depicted in Figure 19 C. IL-4 producing cells were found to be predominant in non-diabetic NOD.Scid mice that were adoptively pre-transferred with X-DT cells and then challenged with DT cells.

Diabetes was first detected in some mice 3 weeks after challenge with Thy 1.1 DT cells. By 12 weeks after Thy 1.1 DT cell challenge, approximately half the mice had developed detectable diabetes. Diabetic mice had lower overall T-cell counts than did non-diabetic mice. The percentage of Thy 1.1 cells in relation to total T-cells was higher in diabetic mice than in non-diabetic mice, although non-diabetic mice had greater total numbers of Thy 1.1 cells than did diabetic mice. Thus, the absolute numbers of diabetogenic T-cells do not appear to be determinative of disease development. Instead, the relative levels of protective and diabetogenic T-cells appears to be important. T-cells from non-diabetic mice expressed higher levels of IL-4 than did T-cells from diabetic mice.

EXAMPLE 20 T-CELLS ISOLATED FROM NON-DIABETOGENIC NOD.SCID MICE ADOPTIVELY TRANSFERRED WITH X-DT CELLS ARE NOT PATHOGENIC

The ability of XLCM cultured cells to regulate the development of diabetes upon transfer of a mixed cell population to a second mouse was examined.

A first population of T-cells was isolated from NOD mice suffering from active diabetes. These cells were cultured in XLCM for 6 days (generating X-DT cells) and then adoptively transferred into NOD.Scid mice. Sixty days later these NOD.Scid mice were challenged by the transfer of uncultured T-cells from diabetic NOD mice (DT cells). Some recipient mice developed diabetes while others were protected. Sixty days after the DT cell challenge, T-cells were isolated from the spleens of the diabetic and non-diabetic mice which had received the first cell population and the challenge. These cells were transferred directly to NOD.Scid mice and the incidence of diabetes in the second set of recipients was monitored. The procedure employed is illustrated in Figure 20 A. The results of this experiment are depicted in Figure 20 B.

T-cells obtained from non-diabetic mice did not cause detectable diabetes in the recipient mice during the 15 week observation period. T-cells from diabetic mice caused diabetes in all recipient mice within 7 weeks after transfer. Thus, XLCM culture is capable of rendering diabetogenic T-cells non-diabetogenic and this effect persists through transplantation into a second animal. Further, XLCM culture is capable of inducing the cultured T-cells to exert a protective effect rendering injected DT cells non-diabetogenic, suppressing diabetes, and this effect persists through transplantation into a second animal.

EXAMPLE 21 THE IMPACT OF CULTURE IN XLCM ON THE DIABETOGENIC AND IDDM PREVENTATIVE TENDENCIES OF ISOLATED CD4 AND CD8 CELLS

Spleens were removed from diabetic NOD mice, and $CD4^+$ cells were isolated according to standard procedures. The isolated $CD4^+$ cells were cultured in HBCM-2 medium containing 5 % XLCM™ in the absence or presence of IL-4 (5 ng/ml) for 4 days. The resultant cell population was adoptively transferred (5 000 000 cells per

mouse) into NOD.Scid mice. A portion of the mice were challenged by the transfer of a population of uncultured diabetogenic cells (DT cells) at 0 or 7 days after the initial transfer of X-DT cells. The mice were observed for 18 weeks and the onset of diabetes was noted. The results of this experiment are depicted in Figure 21 A. The same basic procedure was repeated using isolated $CD8^+$ cells with the effect of DT cell challenge at day 0, week 1, and week 8 examined. The results of this experiment are depicted in Figure 21 B.

The transfer of $CD4^+$ X-DT cells alone lead to the onset of diabetes in approximately 70 % of the mice within 5 weeks of transfer and no further incidence was noted in the 18 week observation period. Where $CD4^+$X-DT cells were cultured in the presence of exogenous IL-4, the rate of diabetes after 5 weeks was approximately 60 %, with no increase during the observation period.

The transfer of $CD8^+$ X-DT cells alone did not lead to the onset of diabetes within the 18 week observation period. Moreover, $CD8^+$ X-DT cells had a protective effect against diabetogenesis by DT "challenge" cells that increased with the time intervals between injection of $CD8^+$ X-DT cells and DT challenge. These results are depicted in Figure 21 B.

While it is not intended that the scope of the invention should be interpreted or limited by any particular theory or postulation of its mode of action, the following discussion is offered for a more complete understanding of the invention as a whole.

It is believed that the maturation from DP to SP thymocytes is initiated by interaction of the TcR on DP thymocytes with peptide bound to MHC molecules expressed on thymic stromal cells. However, since DP thymocytes may differentiate *in vitro* into either $CD4^+CD8^-$ or $CD4^-CD8^+$ SP thymocytes in the absence of thymic stromal cells, the differentiation of DP to SP thymocytes may not strictly require an interaction between TcR/co-receptor and peptide-MHC complexes. In agreement with

these findings, XLCM induces the proliferation and differentiation of DP into $CD4^+CD8^-$ and $CD4^-CD8^+$ SP thymocytes in the absence of thymic stromal cells. Unlike other culture systems in which thymocytes must be activated by stimulation via the TcR/CD3 complex, the differentiation of thymocytes in XLCM does not appear to require such stimulation. XLCM consists of multiple factors, e.g. various cytokines, chemokines and growth factors, which may provide the requisite signals to induce thymocyte proliferation and differentiation and obviate the need for TcR activation. Although IL-2 is present in XLCM and is required for XLCM-driven T cell proliferation, it is unlikely that IL-2 is solely responsible for the support of thymocyte proliferation and differentiation, since addition of exogenous IL-2 to various culture media does not substitute efficiently for the growth stimulatory properties of XLCM.

In the thymus, some DP thymocytes may already be precommitted to the CD4 or CD8 lineage by default, and further selection of a given lineage may be mediated by cytokines. Thus, an alternative explanation for the effect of XLCM on thymocyte maturation is that some of the cytokines present in XLCM may drive precommitted DP thymocytes to differentiate into $CD4^+CD8^-$ or $CD4^-CD8^+$ SP thymocytes by down-regulation of the CD8 or CD4 co-receptors, respectively. This possibility is supported by the observations that CP and IL-4 each alter the pattern of thymocyte differentiation, albeit in an opposite fashion, when added to XLCM. CP stimulates a marked increase in the growth of $CD4^-CD8^+$ SP thymocytes and decrease in the growth of $CD4^+CD8^-$ SP thymocytes, whereas IL-4 enhances the growth of $CD4^+CD8^-$ SP and $CD4^-CD8^-$ DN thymocytes but reduces the growth of $CD4^+CD8^+$ DP and $CD4^-CD8^+$ SP thymocytes. These altered patterns of differentiation were observed for thymocytes from three different mouse strains (C57BL/6, NOD and NOR), and appear to be IL-4-specific as the IL-4 triggered thymocyte differentiation was completely abrogated in the presence of an anti-IL-4 mAb. The finding is consistent with that observed in a 3 day fetal thymus organ culture system, in which IL-4 treatment increases the growth of $CD4^+CD8^-$ SP thymocytes and decreases the growth $CD4^+CD8^+$ DP thymocytes. An important difference between the XLCM and 3 day thymus organ culture systems is that, in XLCM, IL-4 directly acts on DP thymocytes in the absence of

stromal cells to deliver signals for $CD4^+$ SP thymocyte differentiation. Thus, in XLCM, CP and IL-4 can provide signals that either up- or down-regulate the expression of CD8 and CD4, respectively.

Although IL-4 stimulates the growth of $CD4^+CD8^-$ SP thymocytes, the mechanism by which IL-4 regulates the positive selection of these SP thymocytes remains unclear. In accordance with the correlation between increased surface expression of CD69 on thymocytes and positive thymic selection, we found that IL-4 significantly enhances CD69 expression on C57BL/6, NOD and NOR thymocytes cultured in XLCM, and on $CD4^+CD8^+$ DP thymocytes in particular. This result, together with the finding that anti-IL-4 mAb blocks the IL-4 induced elevation of CD69 expression on DP thymocytes, suggests that DP thymocytes are the major cell subpopulation in the thymus that is activated by IL-4.

Several strain-related differences were detected in the proliferation and differentiation of NOD and C57BL/6 thymocytes cultured in XLCM. First, while C57BL/6 $CD4^-CD8^+$ SP thymocytes were predominant in the initial 4 day cultures, NOD $CD4^+CD8^-$ SP thymocytes were more abundant in these cultures. Second, a higher percentage of $CD69^+$ cells was obtained after a 4-6 day culture of NOD than C57BL/6 thymocytes. Third, addition of IL-4 to XLCM increased the frequency of IL-4- and IL-10-producing C57BL/6 but not NOD thymocytes. Interestingly, thymocytes from NOR mice, which are much more genetically similar to NOD than C57BL/6 mice, displayed a pattern of differentiation akin to that of NOD thymocytes. These results suggest that both genetic background and environmental factors in the thymus determine thymocyte differentiation.

Thymocytes differentiate in XLCM to produce IL-2, IL-4, IL-10 and IFN-γ, and the percentages of IL-4 and IL-10 producing thymocytes were highest in these cultures. Since XLCM contains little IL-4, other factor(s) present in XLCM likely promote IL-4 secretion. Exogenous IL-4 stimulates the synthesis and secretion of IL-4 and IL-10 by thymocytes cultured in XLCM. In contrast, virtually complete inhibition

of IL-4 and IL-10 production is observed upon addition of CP, even though CP simultaneously promotes thymocyte proliferation. These results suggest that the ability of thymocytes to produce cytokines may be determined by environmental factors in the thymus, which in turn may influence thymocyte differentiation, as suggested above.

In conclusion, XLCM is a unique conditioned medium capable of inducing the proliferation and differentiation of specific T-cell subtypes *in vitro* in the absence of thymic stromal cells, and therefore represents an excellent growth medium for exploring factors that have an important impact on thymocyte differentiation. By using this process, we have obtained additional supportive evidence that IL-4 plays an important role in thymocyte differentiation, which is characterized by the differentiation of $CD4^+CD8^-$ SP thymocytes and an increase in surface CD69 expression on DP thymocytes. XLCM may be used to generate regulatory T cells, which may have important implications for cell therapy of several immunological disorders, including HIV-1 infection, cancer and autoimmune diseases including IDDM.

CLAIMS:

1. A process for *ex vivo* proliferation of T-cells and differentiation thereof to produce cell populations enriched in pre-selected sub-populations of T-lymphocytes, which comprises *ex vivo* culturing T-cells in a culture medium containing a cell suspension conditioned culture medium CM, and under appropriate cell proliferation conditions, and obtaining in the culture medium a cell population enriched in the preselected sub-population.

2. The process of claim 1 wherein the culture medium is an aqueous culture medium containing a minor proportion by volume of said conditioned culture medium CM.

3. The process of claim 2 wherein the conditioned culture medium CM is the aqueous liquid product resulting from the culturing therein of a cell population comprising peripheral blood cells, umbilical cord blood cells, bone marrow cells, mixtures of two or more types of such cells, or fractions or mixed fractions of such types of cells, in the presence of an inducing agent which includes at least one plant mitogen.

4. The process of claim 3 wherein the medium CM derives from culturing said cells in the presence of at least one plant mitogen selected from concanavalin A, phytohemagglutinin, mezerein and tetradecanoyl phorbol acetate.

5. The process of claim 4 wherein the plant mitogens used in preparation of the medium CM are concanavalin A and mezerein.

6. The process of claim 5 wherein a starting population of DP thymoyctes CD4+ CD8+ T-cells is cultured in the presence of conditioned medium CM to produce cell populations enriched in predetermined single positive CD4+ T-cells, CD8+ T-cells or sub-populations of such cells.

7. The process of claim 6 wherein DP thymocytes are cultured in the presence of

conditioned medium CM and in the presence of added exogenous IL-4 to promote differentiation thereof to SP thymocytes.

8. The process of claim 7 wherein the culturing is conducted in the substantial absence of thymic stromal cells.

9. The process of claim 5 wherein T-cells are cultured in a culture medium containing conditioned medium CM to produce a cell population enriched in IL-4 producing cells.

10. The process of claim 5 wherein self-reactive T-cells are cultured in a culture medium containing conditioned medium CM to produce a cell population with a reduced level of self-reactivity.

11. The process of claim 5 wherein T-cells are cultured in a culture medium containing conditioned medium CM to produce a cell population capable of delaying the onset of autoimmune diabetes when introduced into an MHC compatible patient.

12. The process of claim 9 wherein the starting T-cells are immature T-cells.

13. The process of claim 9 wherein the starting T-cells are mature T-cells.

14. The process of claim 9 wherein the culture medium also contains exogenous IL-4.

15. An IL-4 secreting population of mammalian T-cells containing a substantial proportion of a sub-population of Th2-like cells exhibiting a Th2-like cell cytokine production profile, said IL-4 secreting T-cell population having been prepared by cell culturing of a starting cell population containing T-cells, said starting population having a lower Th2-like cell cytokine production profile said than said IL-4 secreting T-cell population.

16. An IL-4 secreting cell population as defined in claim15, having a higher Th2-like cell cytokine production profile than its precursor cell population before culturing thereof in conditioned medium CM described herein.

17. An IL-4 secreting cell population as defined in claim 16, having a higher Th2-like cell cytokine production profile than its precursor cell population before culturing in the presence of XLCM.

18. A process of enhancing the protection of a mammal against the onset and/or development, or alleviating the symptoms, of a Th1/Th2 related cytokine disorder, which comprises administering to the mammal a population of MHC compatible T-cells.

19. The process of claim 18 wherein the cell population is obtained by the process of claim 10 or claim 11

20. The process of claim 18 wherein the cell population is as claimed in 14, claim 15 or claim 16.

21. The process of claim 18 wherein the enriched cell population is obtained by a process as claimed in claim 9.

22. The process of claim18, claim 19, claim 20 or claim 21, wherein the process is a T-cell mediated autoimmune disease.

23. The process of claim 21 wherein the autoimmune disease is IDDM.

24. The use of a population of cells produced by the process of claim 9, claim 10 or claim 11 in the preparation of a medicament for treating an MHC compatible subject against the onset and/or development of an autoimmune disease.

25. Use of a population of a self reactivity suppressing mammalian T-cells in treating

an MHC compatible mammal for protection against the onset and/or development of, or alleviation of the symptoms of, a T-cell mediated autoimmune disorder.

26. Use of a population of mammalian T-cells enriched in IL-4 producing cell sub-population, in treating an MHC compatible mammal for protection against the onset and/or development of, or alleviation of the symptoms of, a Th1/Th2 related cytokine response disorder.

27. Use according to claim 26 wherein the disorder is an autoimmune disease.

28. Use according to claim 26 wherein the disorder is IDDM.

29. Use according to claim 26, claim 27 or claim 28 wherein the cell population is as defined in claim 15, claim 16 or claim 17.

30. Use according to claim 26 wherein the IL-4 producing enriched cell sub-population has been obtained by a process as claimed in claim 9.

Figure 1. XLCM™ Supports Thymocyte Proliferation

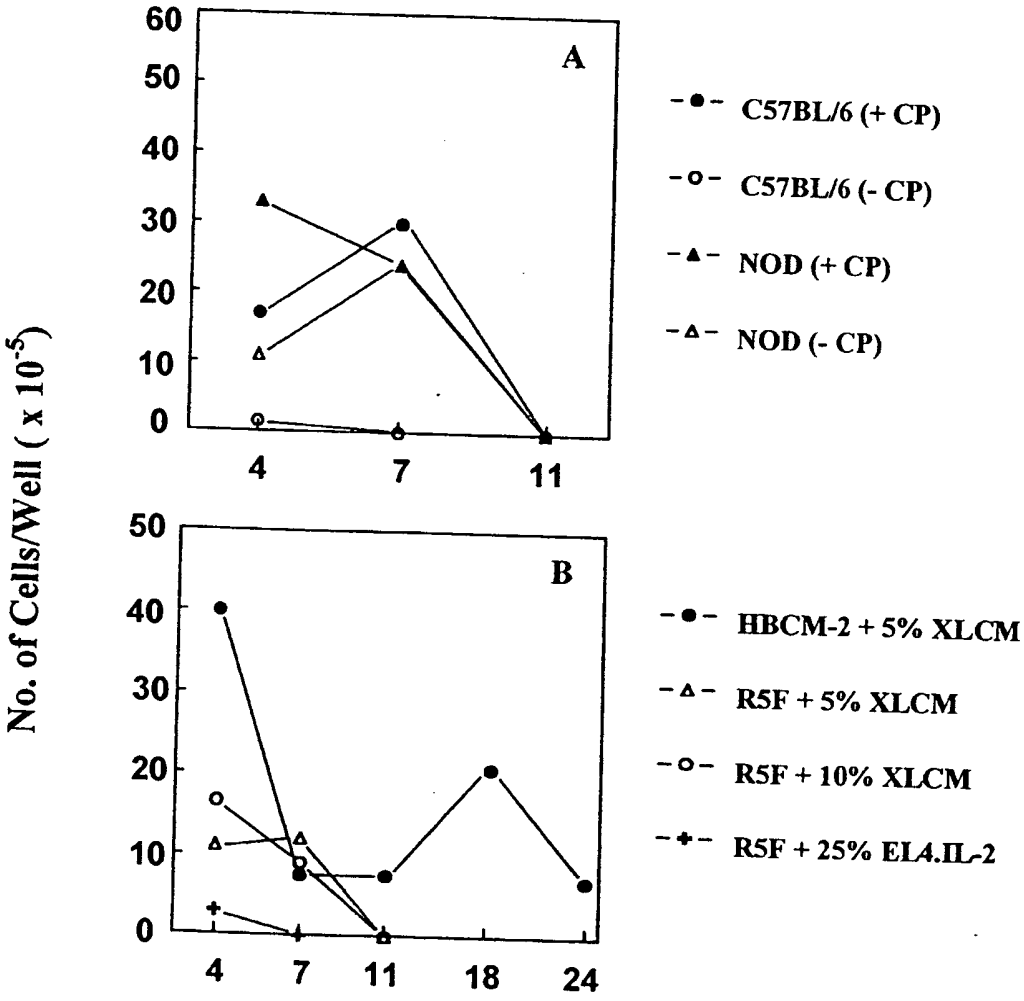
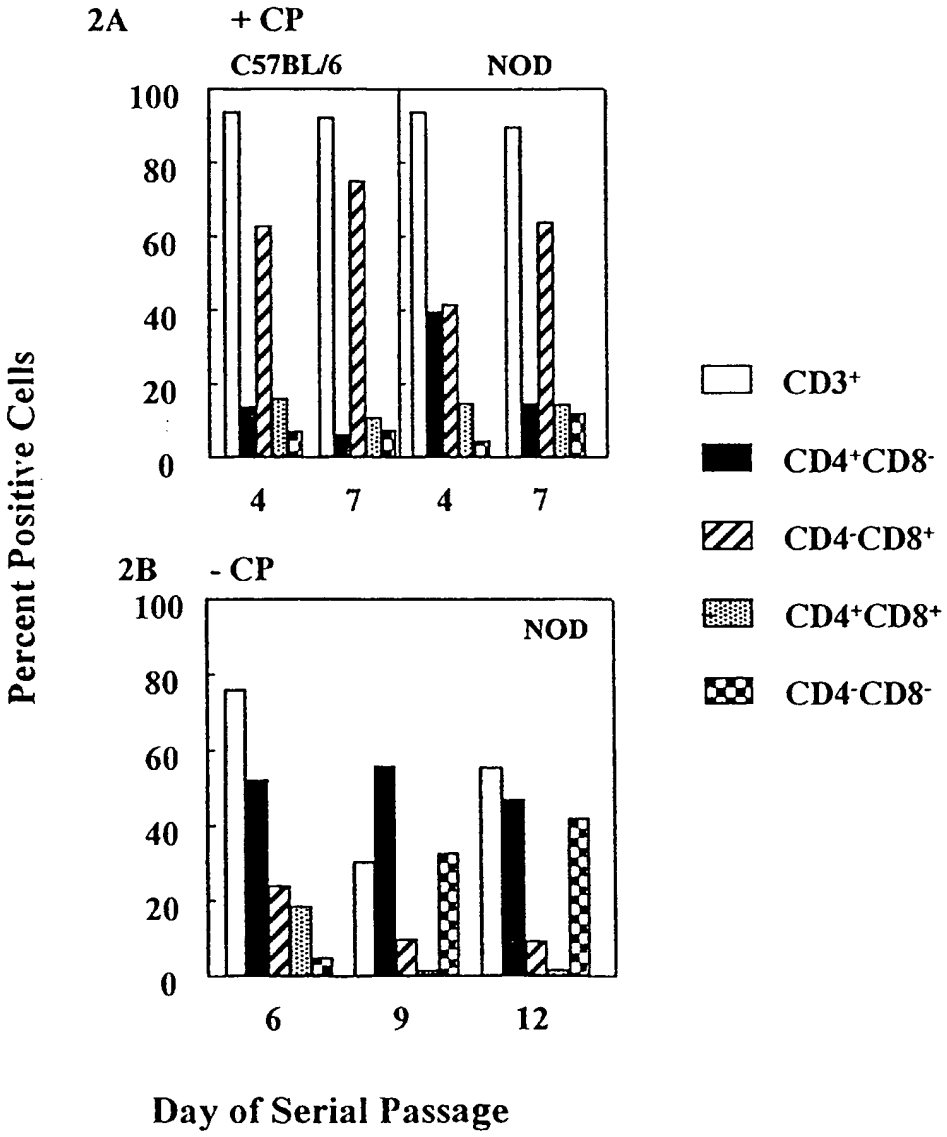


Figure 2 XLCM™ Promotes Thymocyte Differentiation



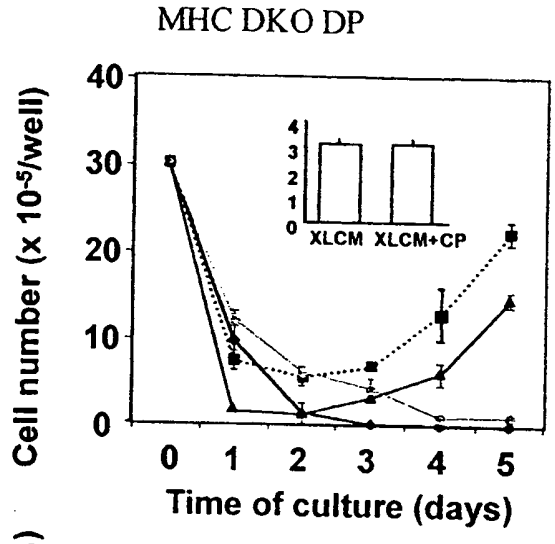


Figure 2C

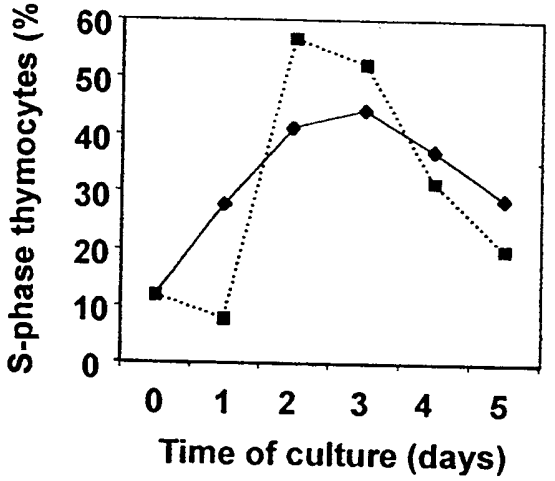


Figure 2D

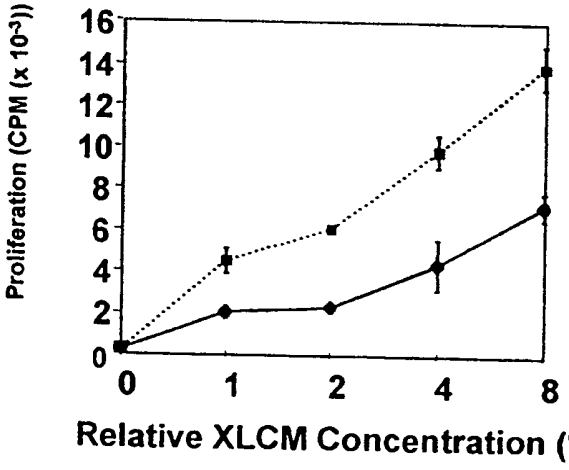


Figure 2E

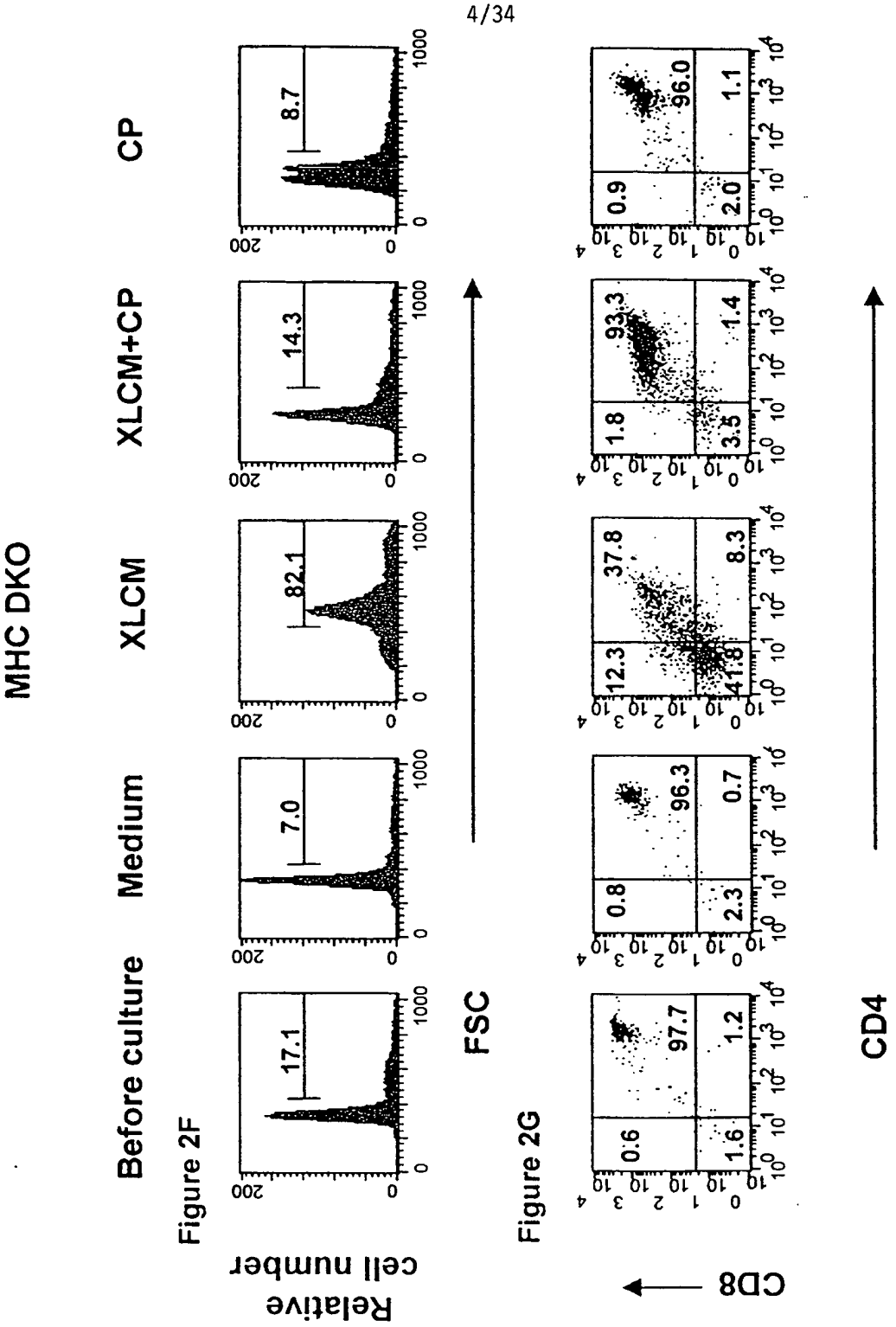


Figure 2F

Figure 2G

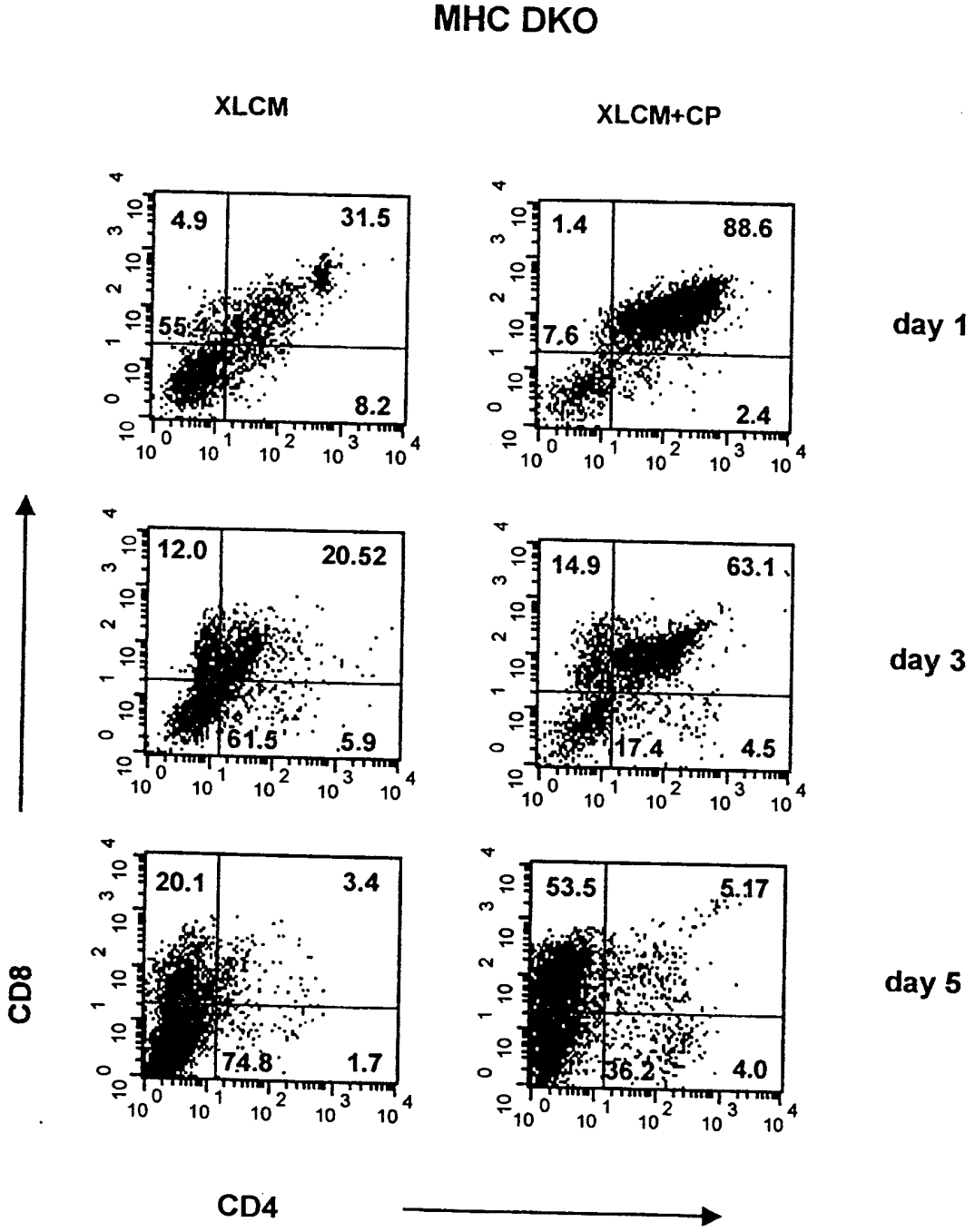

MHC DKO
XLCM
XLCM+CP
4.9
31.5
55.4
8.2
1.4
88.6
7.6
2.4
day 1
12.0
20.52
61.5
5.9
14.9
63.1
17.4
4.5
day 3
20.1
3.4
74.8
1.7
53.5
5.17
36.2
4.0
day 5
CD8
CD4

Figure 2H

Figure 3A

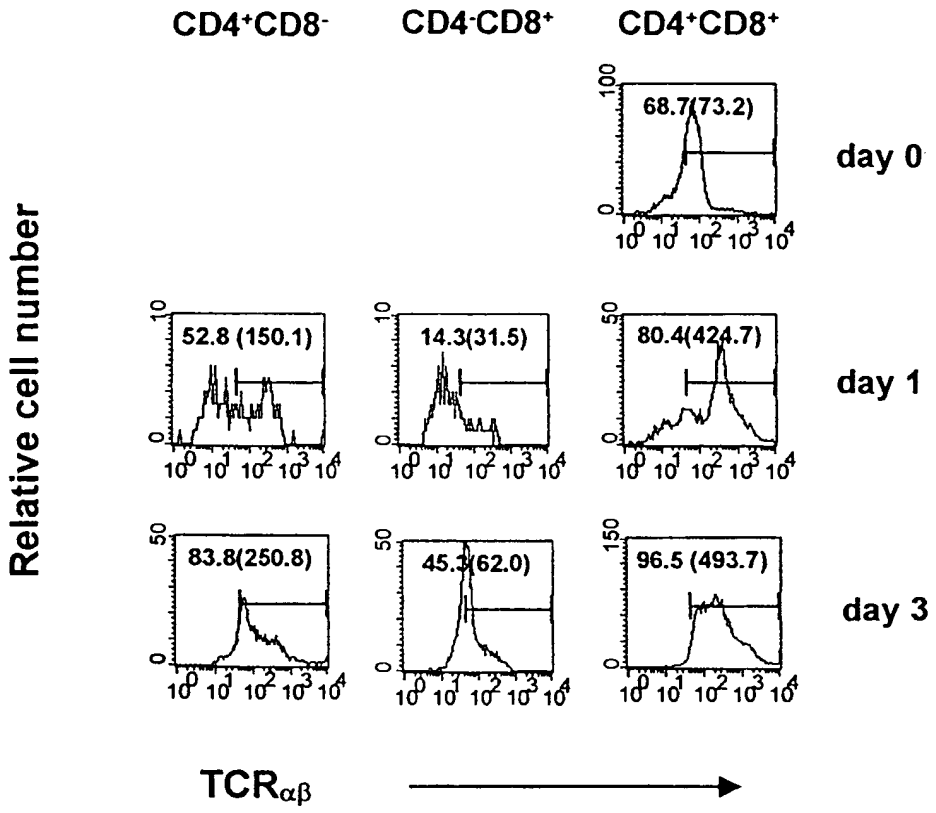

CD4+CD8-
CD4-CD8+
CD4+CD8+
68.7(73.2)
day 0
52.8 (150.1)
14.3(31.5)
80.4(424.7)
day 1
83.8(250.8)
45.3(62.0)
96.5 (493.7)
day 3
Relative cell number
TCRαβ

Figure 3B

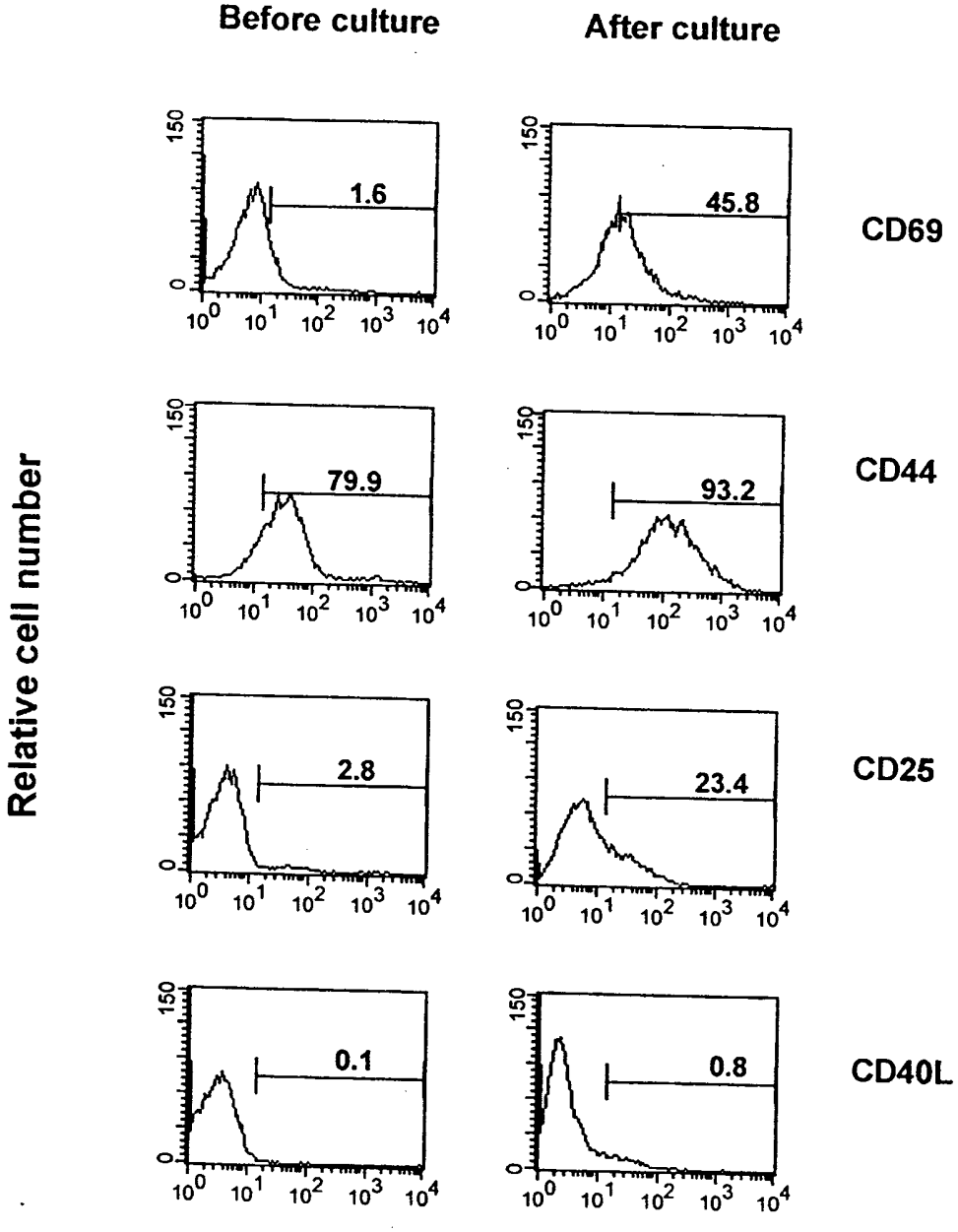

Before culture
After culture
1.6
45.8
CD69
79.9
93.2
CD44
2.8
23.4
CD25
0.1
0.8
CD40L
Relative cell number
Fluorescence intensity

Figure 4A

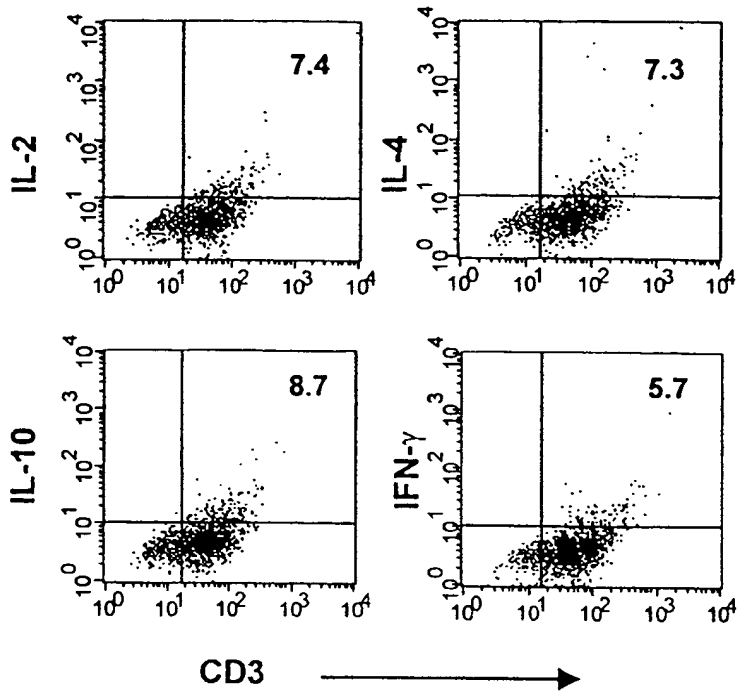


Figure 4B

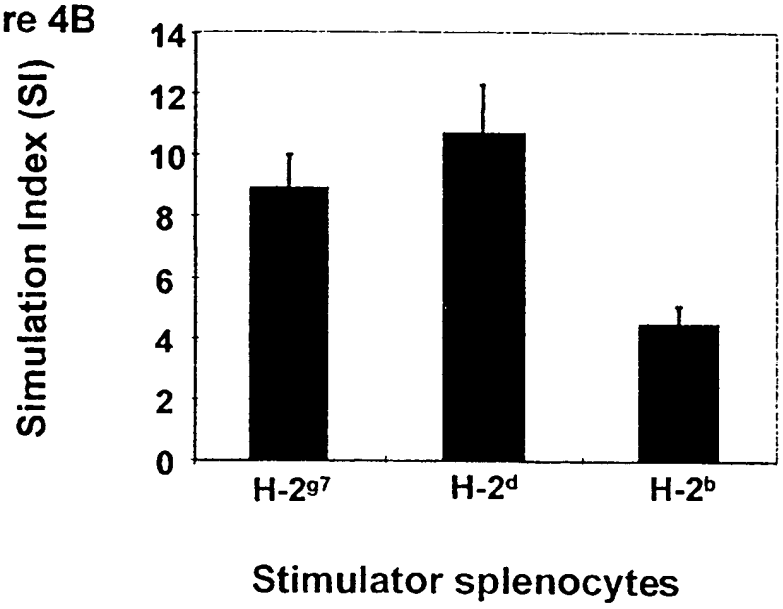
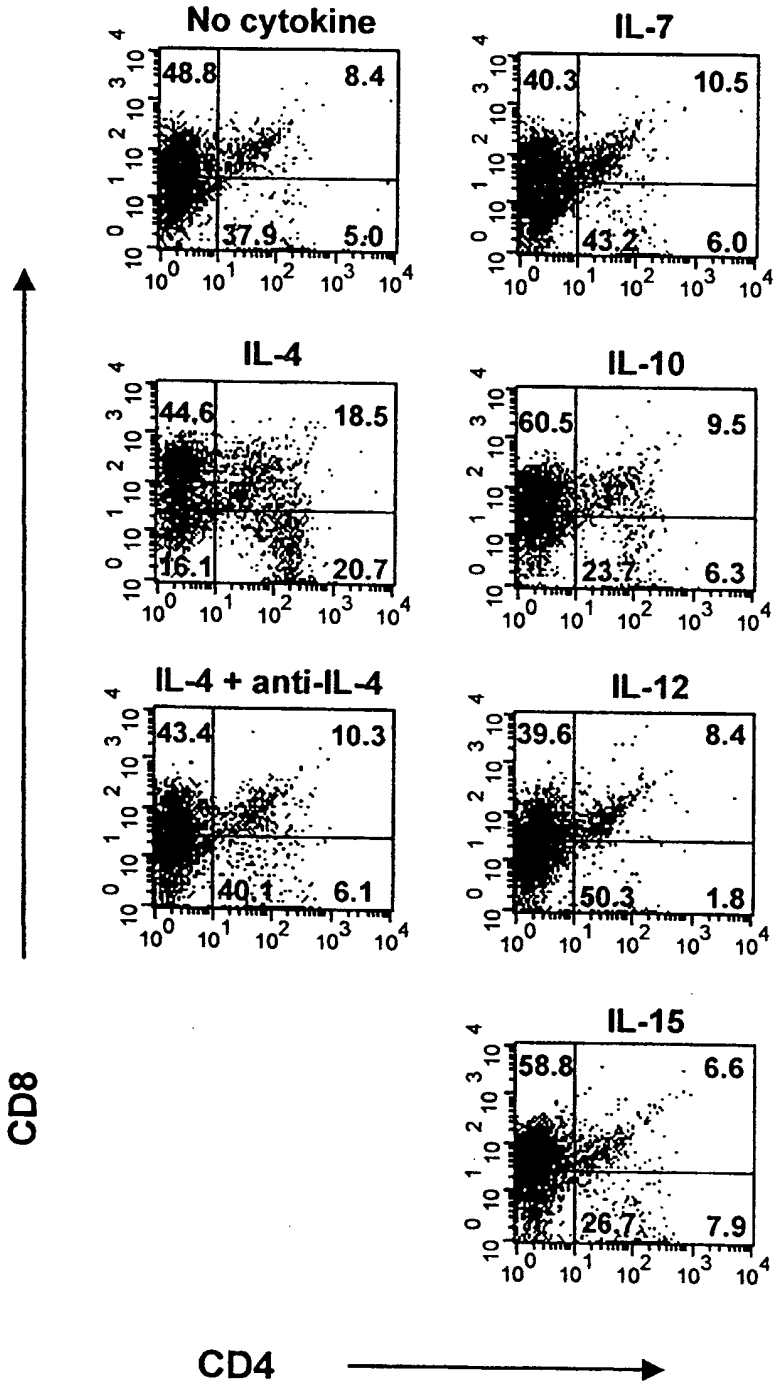


Figure 5A


No cytokine
48.8
8.4
37.9
5.0
IL-7
40.3
10.5
43.2
6.0
IL-4
44.6
18.5
16.1
20.7
IL-10
60.5
9.5
23.7
6.3
IL-4 + anti-IL-4
43.4
10.3
40.1
6.1
IL-12
39.6
8.4
50.3
1.8
IL-15
58.8
6.6
26.7
7.9
CD8
CD4

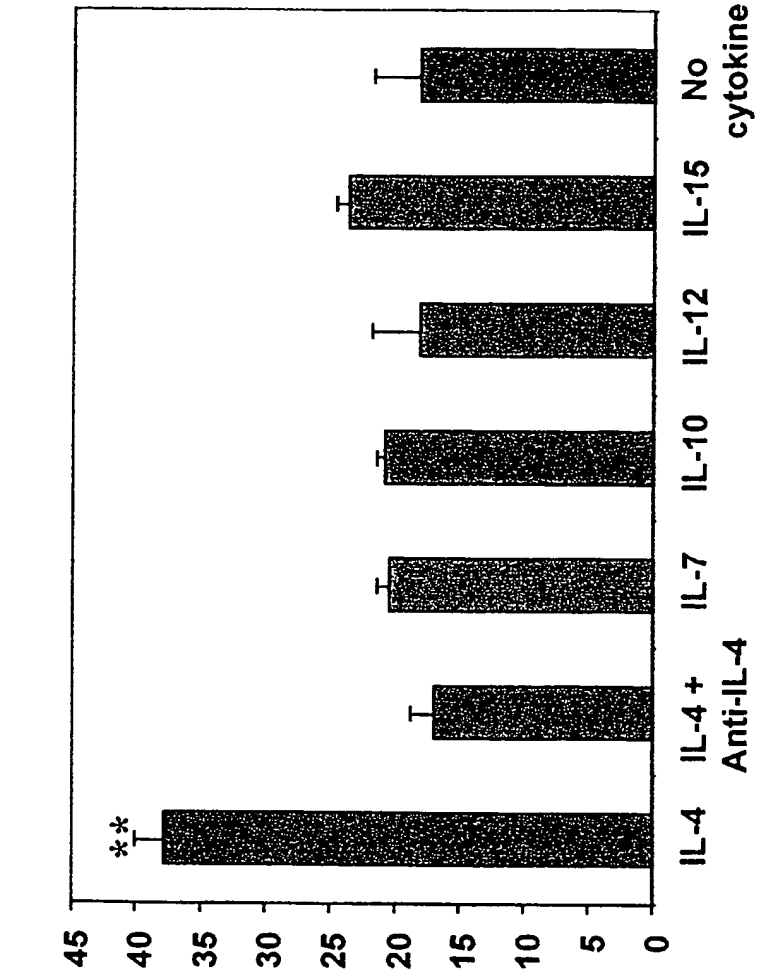

B
Cell number (x 10-5/well)
45
40
35
30
25
20
15
10
5
0
**
IL-4
IL-4 + Anti-IL-4
IL-7
IL-10
IL-12
IL-15
No cytokine

Figure 5B

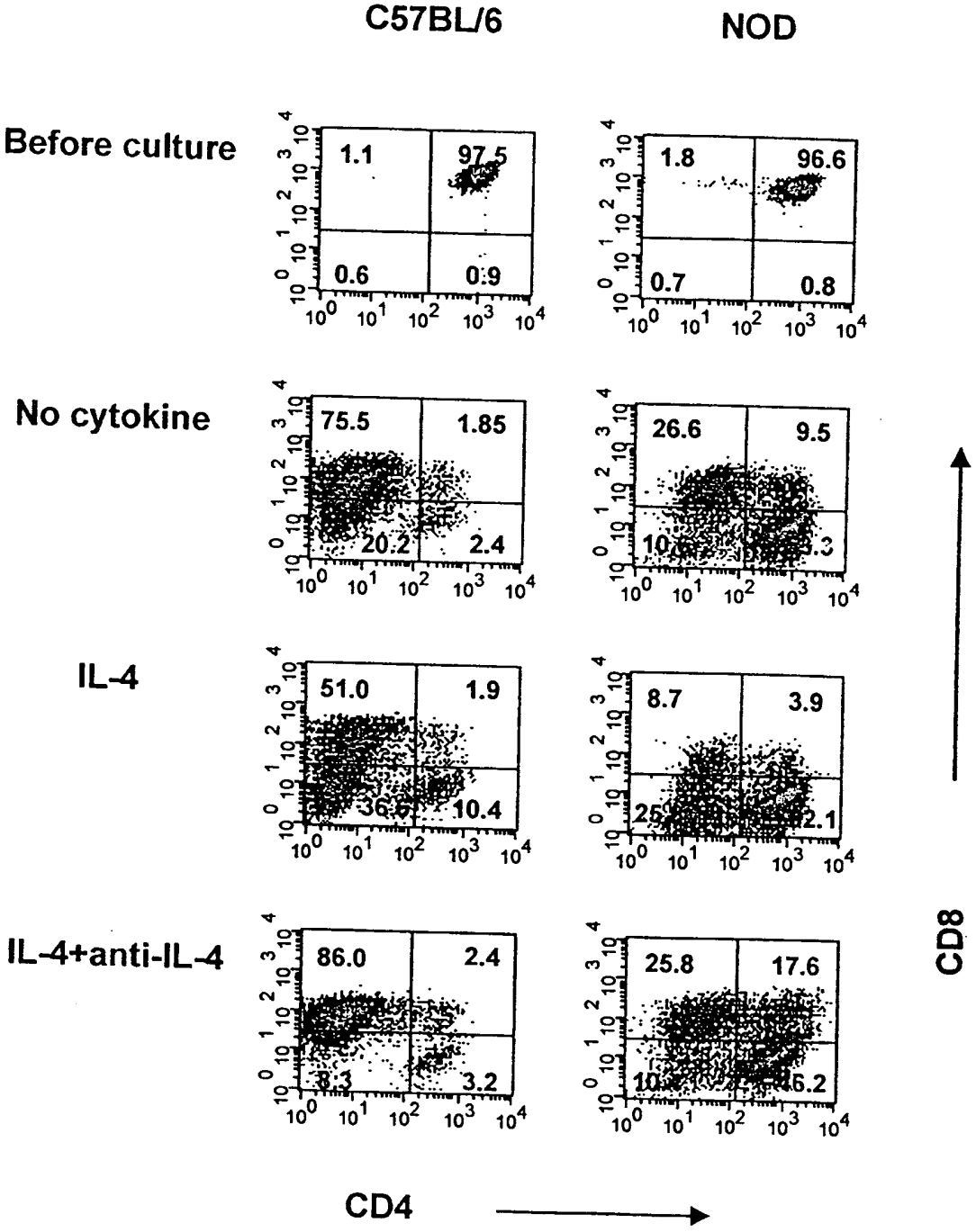

C57BL/6
NOD
Before culture
No cytokine
IL-4
IL-4+anti-IL-4
1.1
97.5
0.6
0.9
1.8
96.6
0.7
0.8
75.5
1.85
20.2
2.4
26.6
9.5
51.0
1.9
36.6
10.4
8.7
3.9
86.0
2.4
3.2
25.8
17.6
CD4
CD8

Figure 6

Figure 7A IL-4 Upregulates CD69 Expression

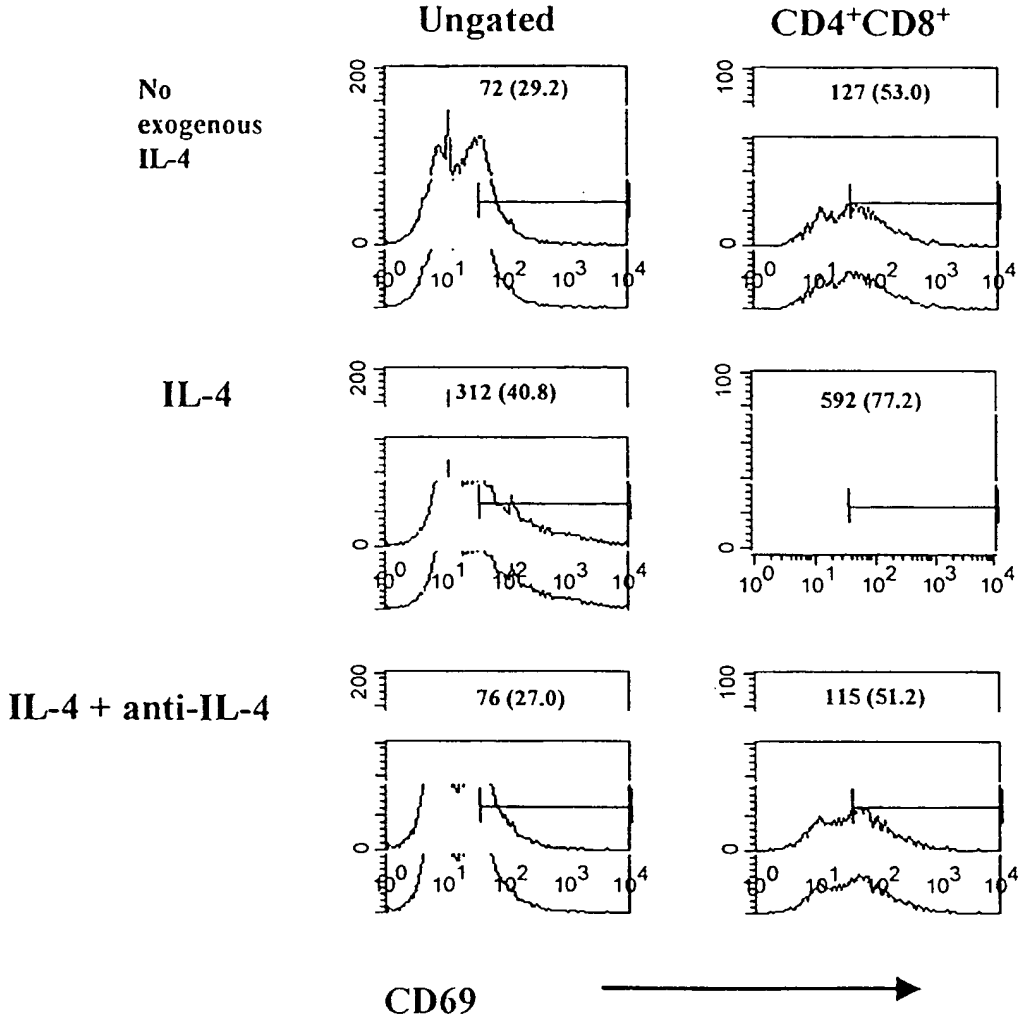


Figure 7B IL-4 Upregulates CD69 Expression

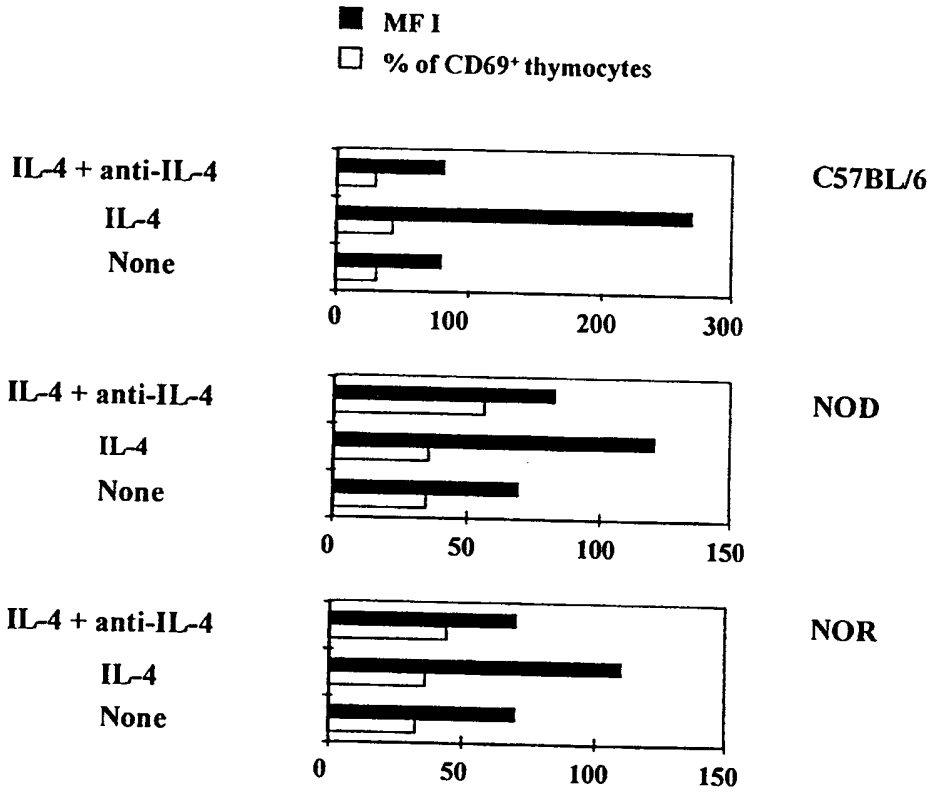


Figure 8A

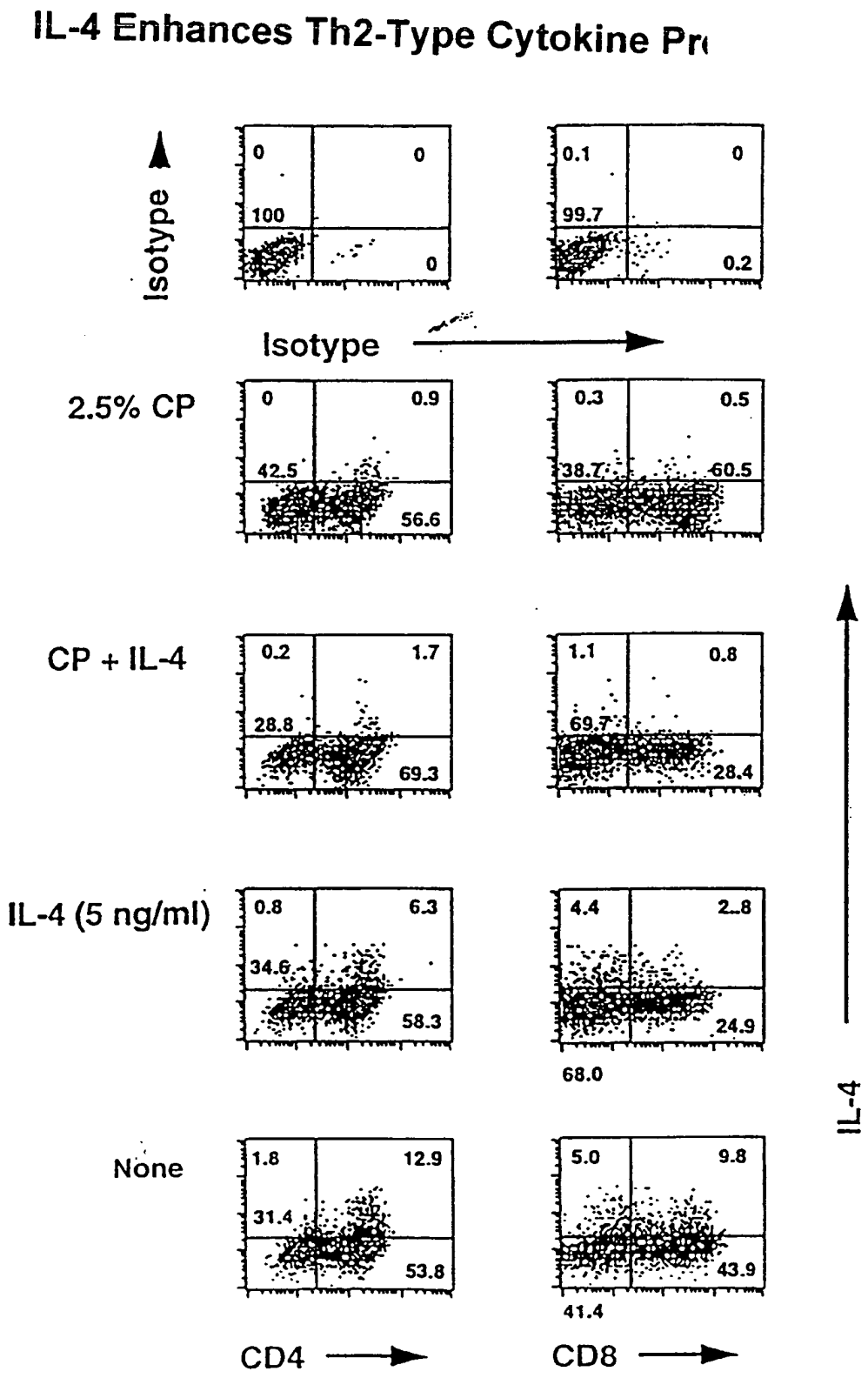

IL-4 Enhances Th2-Type Cytokine Pr
Isotype
Isotype
2.5% CP
CP + IL-4
IL-4 (5 ng/ml)
None
0
0
100
0
0.1
0
99.7
0.2
0
0.9
42.5
56.6
0.3
0.5
38.7
60.5
0.2
1.7
28.8
69.3
1.1
0.8
69.7
28.4
0.8
6.3
34.6
58.3
4.4
2.8
24.9
68.0
1.8
12.9
31.4
53.8
5.0
9.8
43.9
41.4
IL-4
CD4
CD8

Figure 8B IL-4 Enhances Th2-Type Cytokine Production

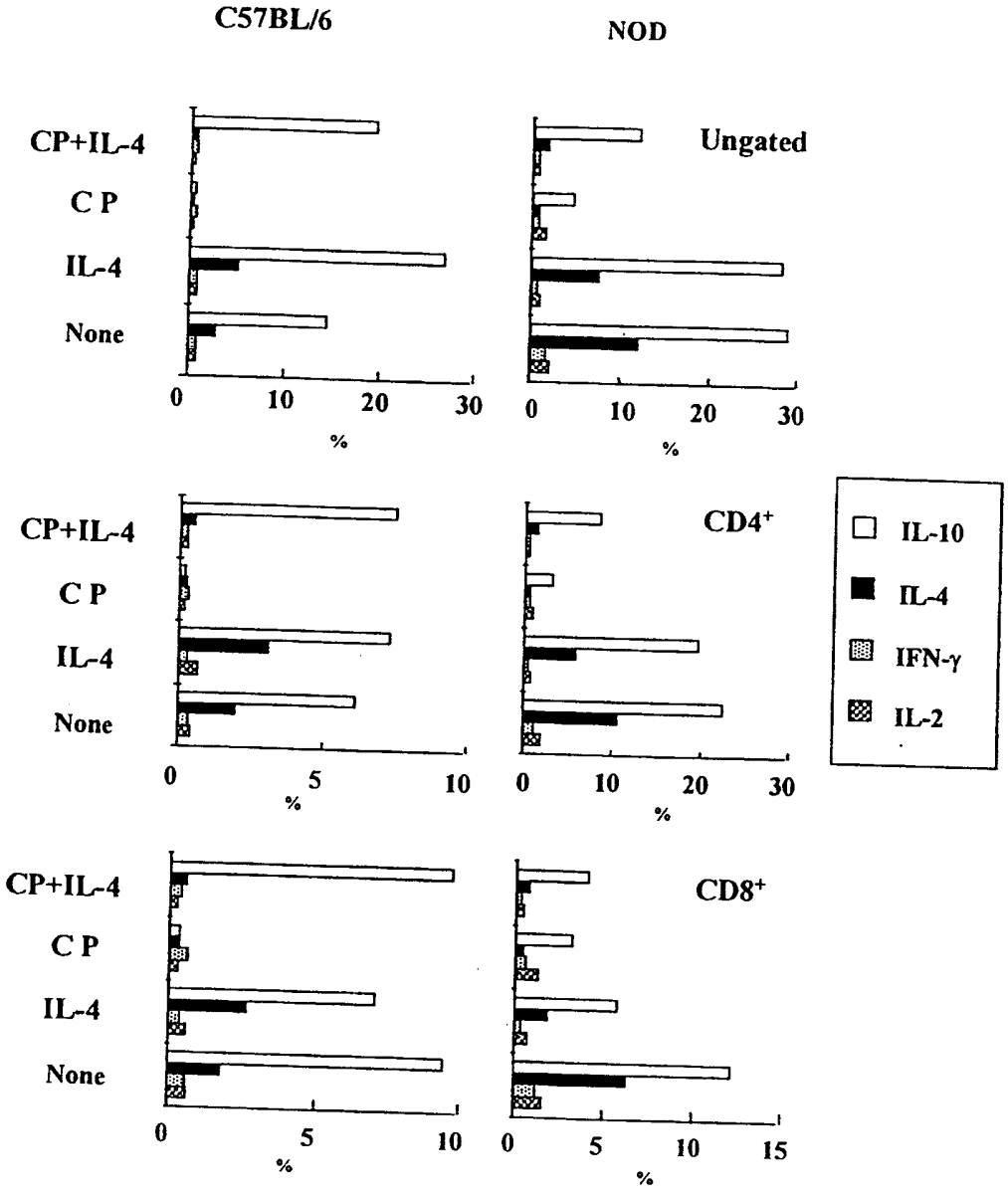


Figure 9 XLCM™ Stimulates Splenic T Cell Proliferation

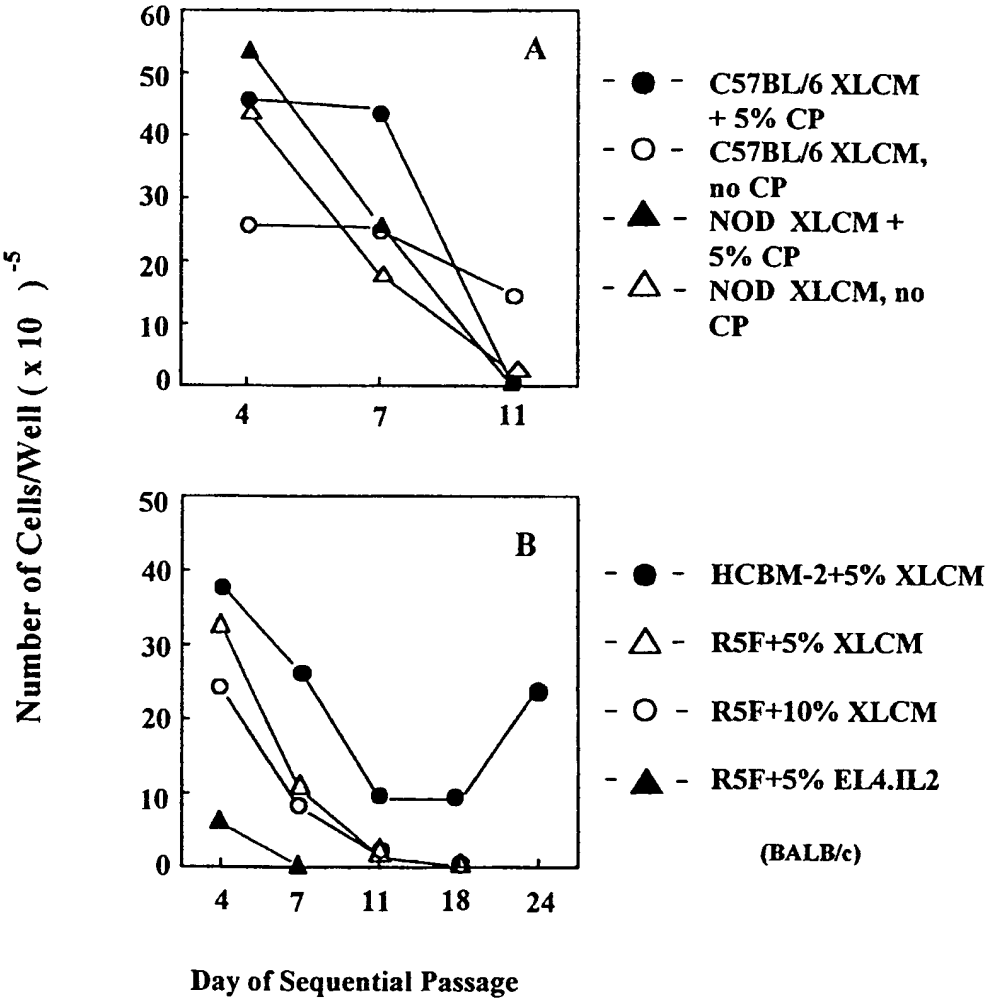


Figure 10 Phenotype of Splenocytes Expanded in XLCM™

Figure 10A

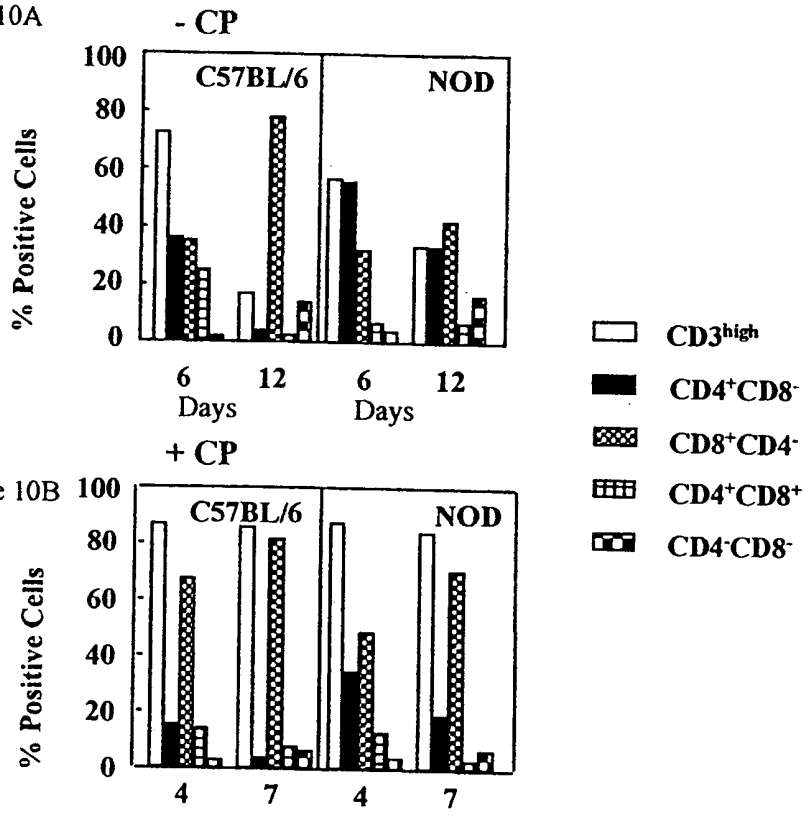


Figure 10B

Days of Sequential Passage

Figure 11 IL-4 Enhances Th2-Like Cells

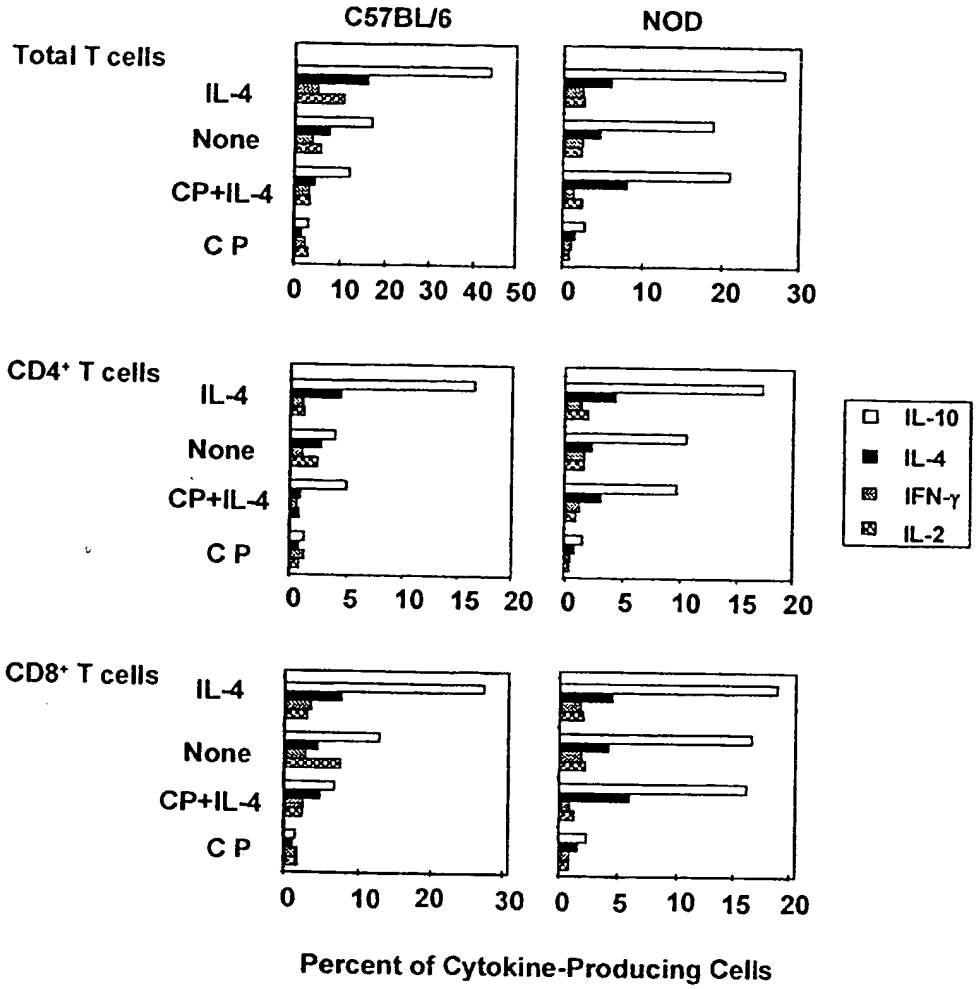


Figure 12 Th2 Cytokine Profile Promotion

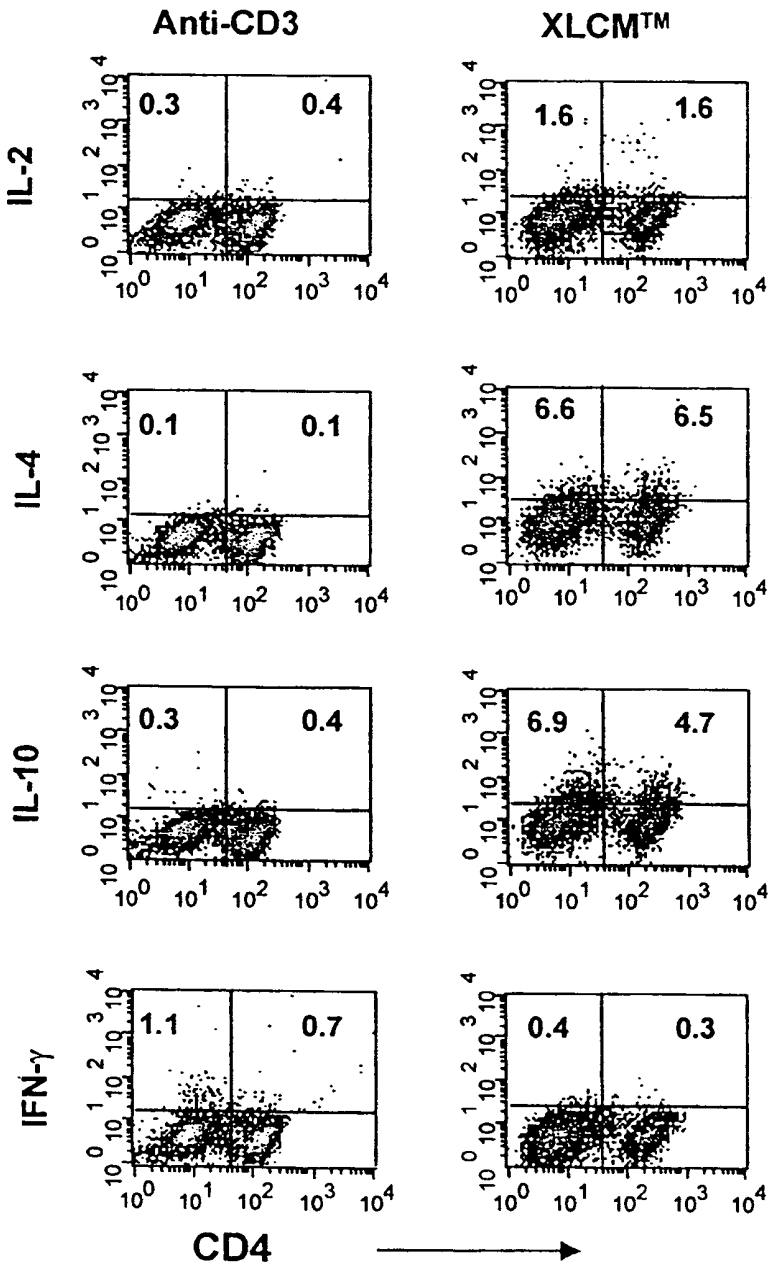


Figure 13 Reduced Diabetogenesis

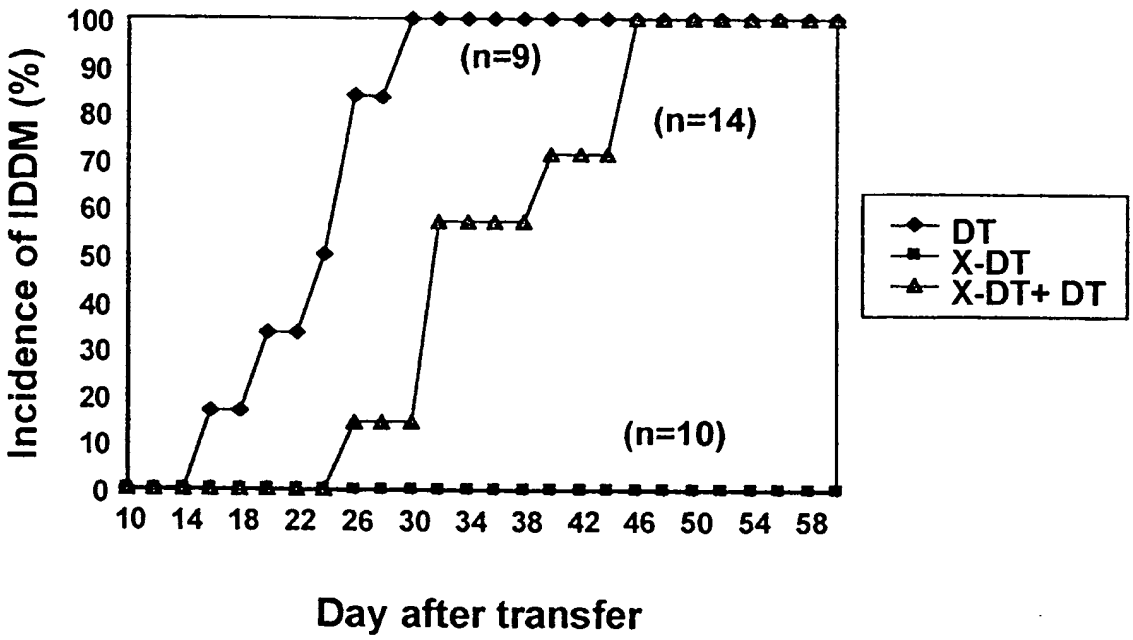


Figure 14 Kinetics of Protection

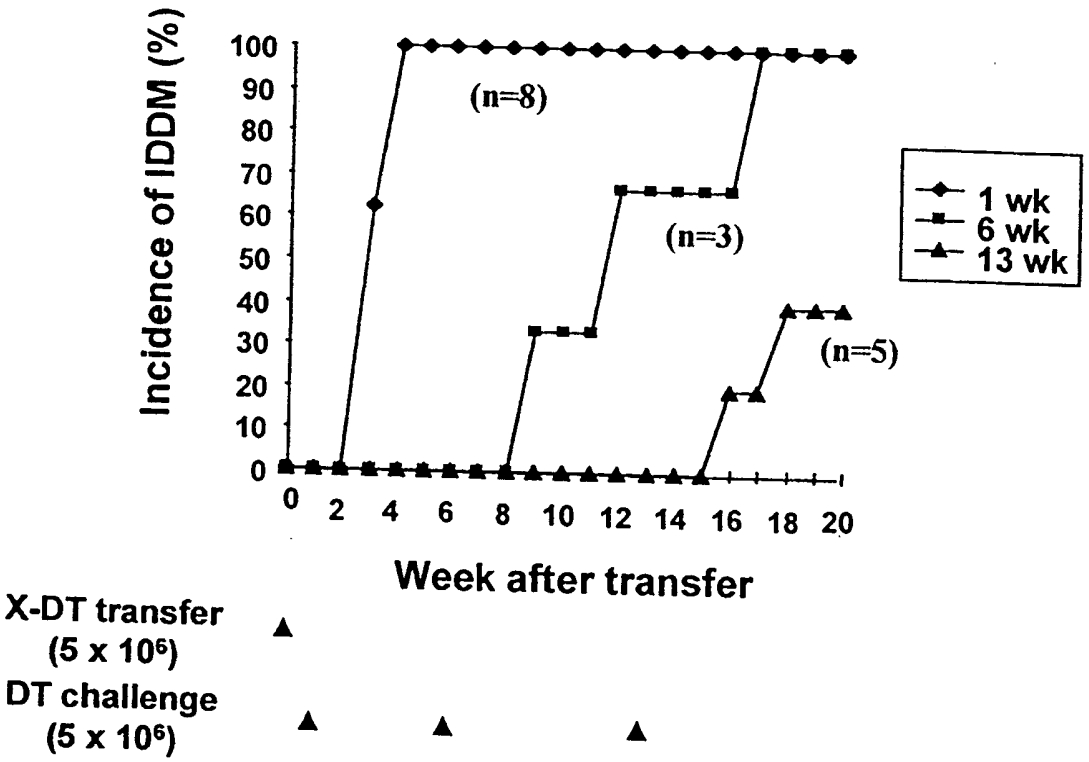


Figure 15A Accessory Cell Culture and IDDM

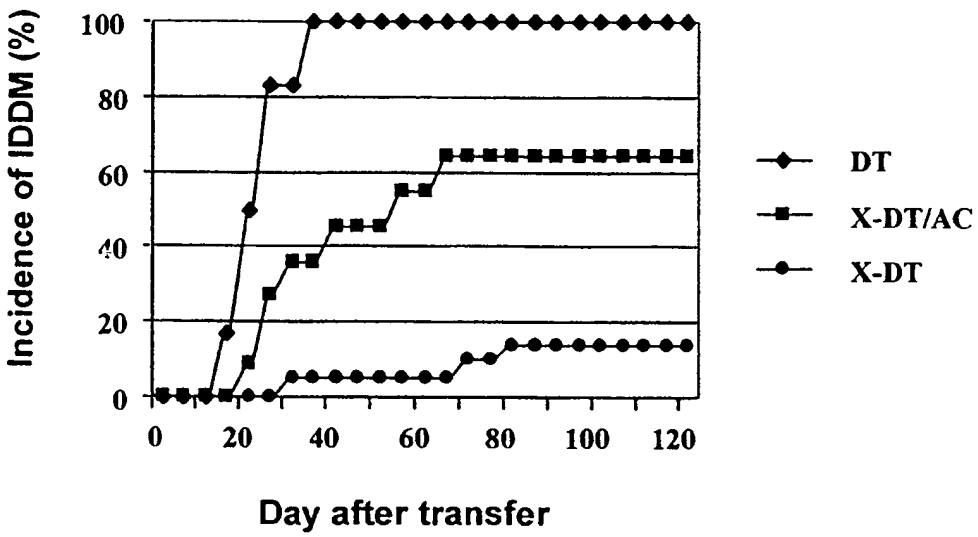


Figure 15B Accessory Cell Culture and Cytokine Production

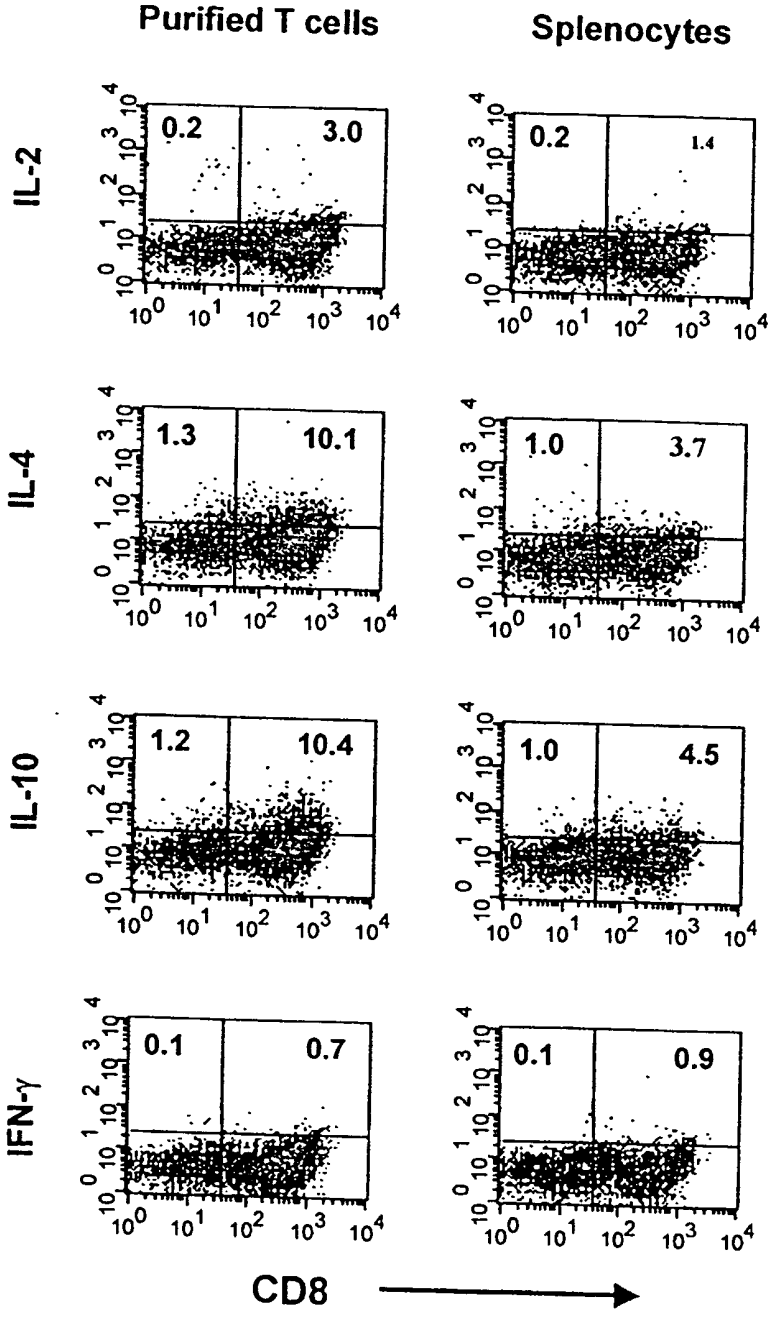


Figure 15C Accessory Cells and CD4, CD8 Expression

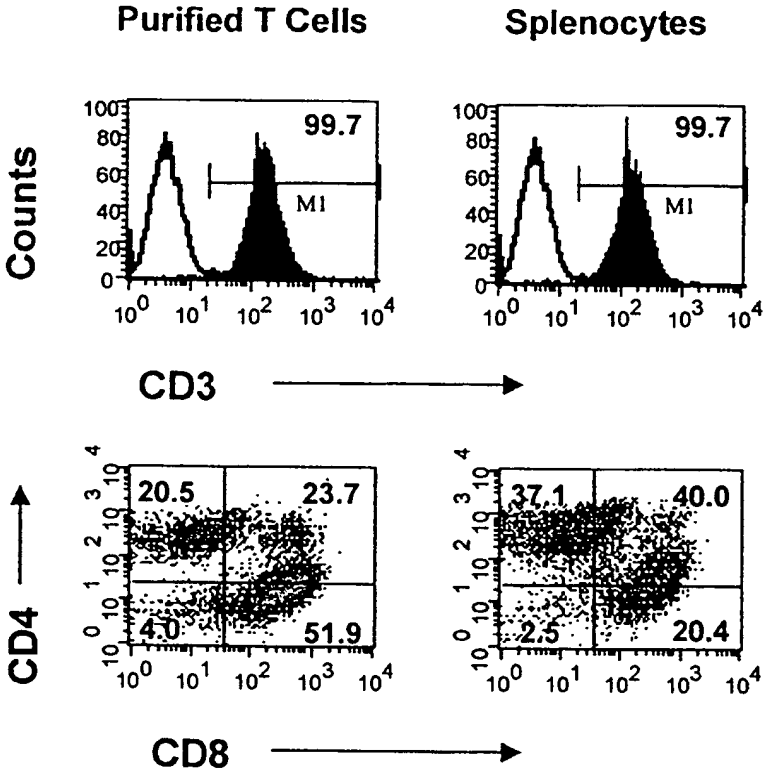


Figure 16 Repopulation and Proliferation of X-DT Cells in NOD.Scid Mice

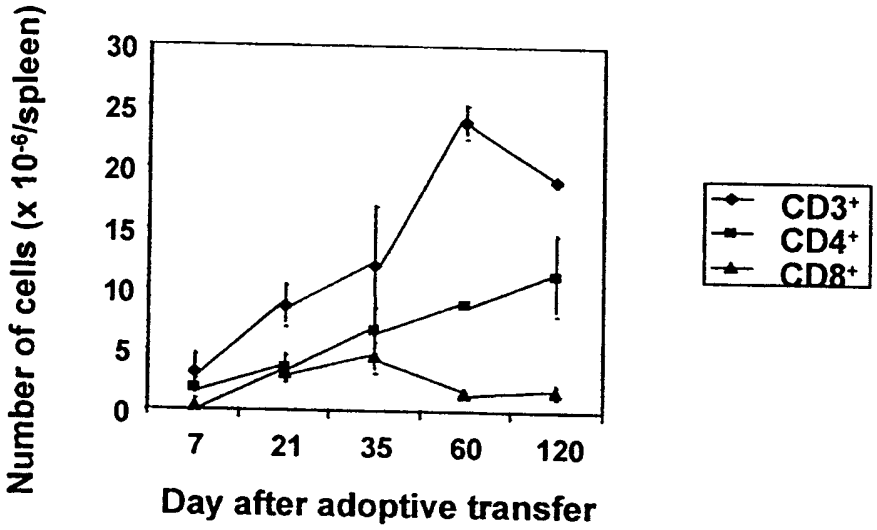


Figure 17 Cytokine Production by Spleen T Cells

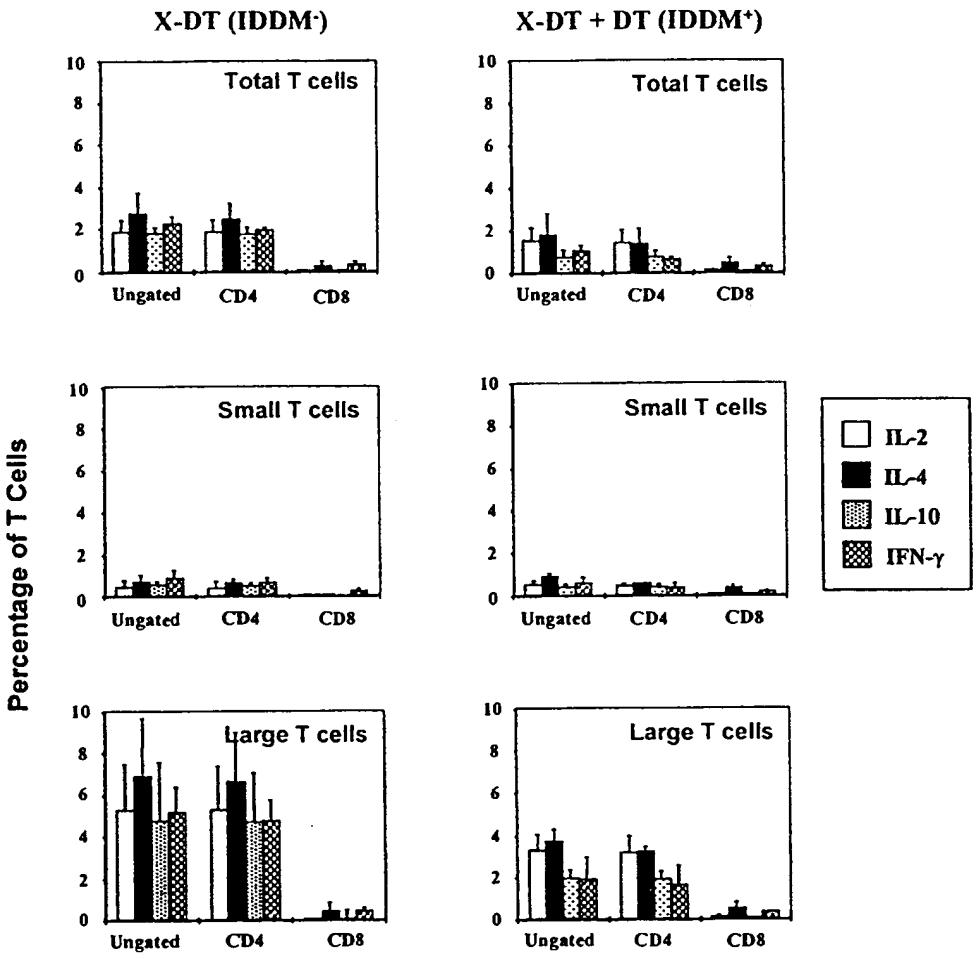


Figure 18 Cytokine Production by Mesenteric Lymph Node T Cells

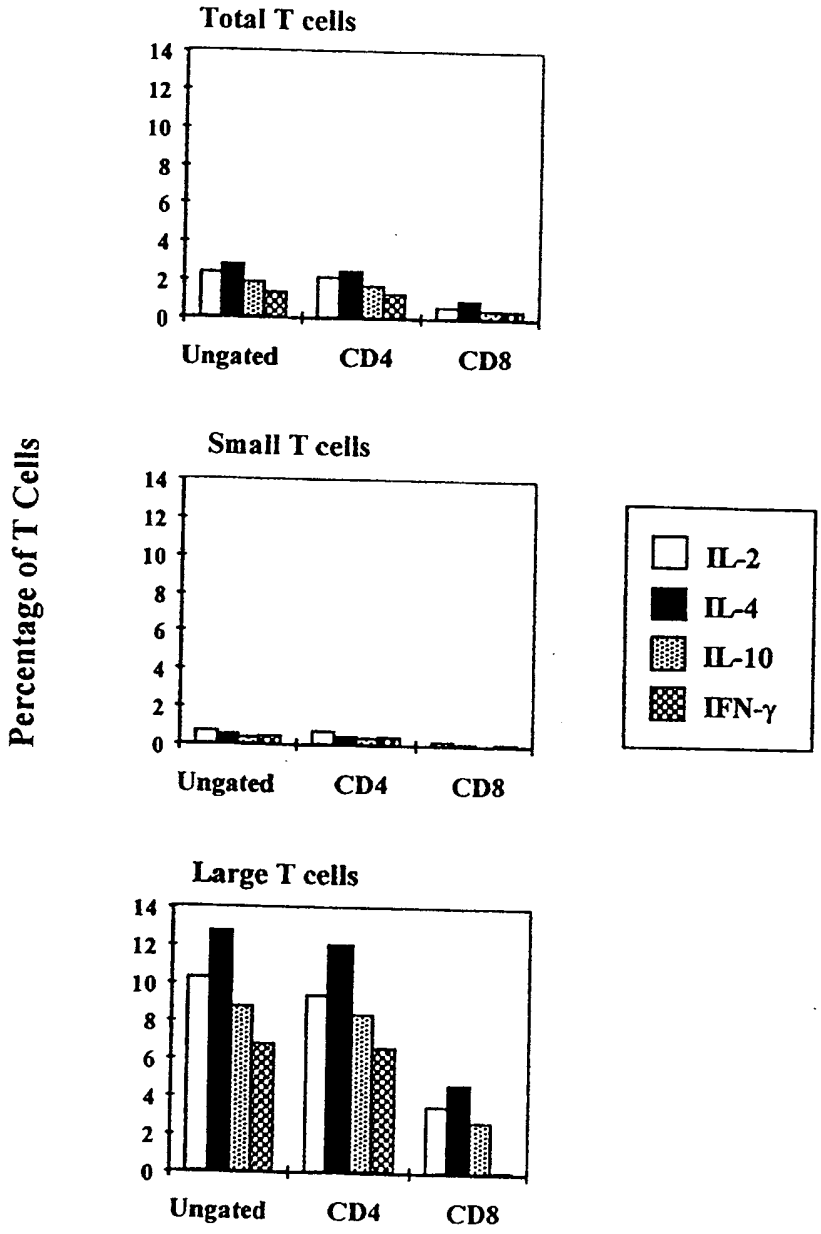


Figure 19A Resistance to DT Cell Challenge

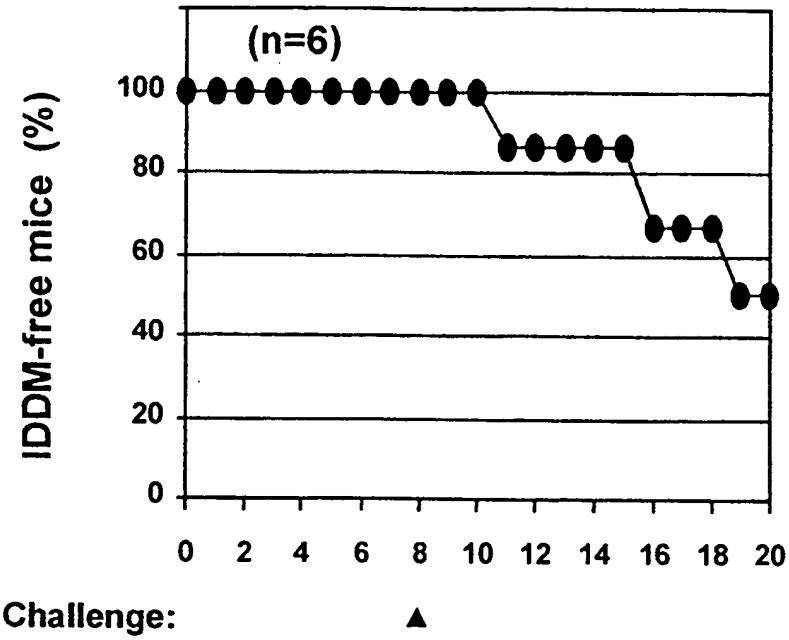


Figure 19B Repopulation of DT Cells

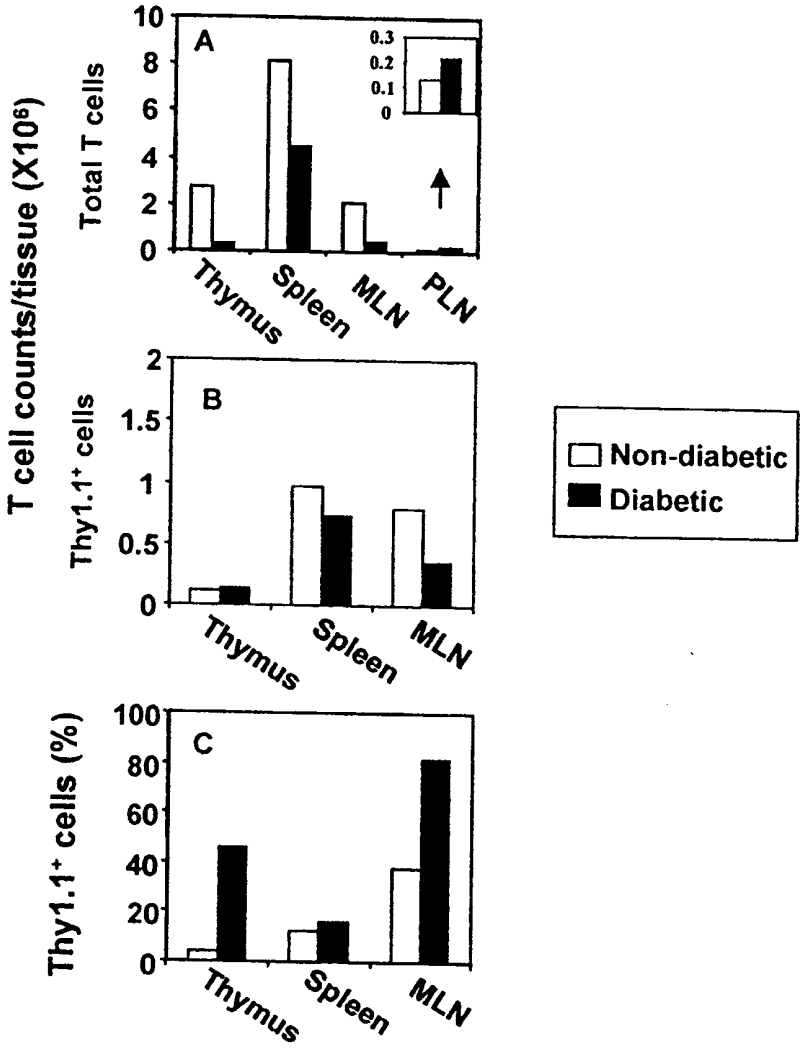


Figure 19C IL-4 Producing Cells and Diabetes

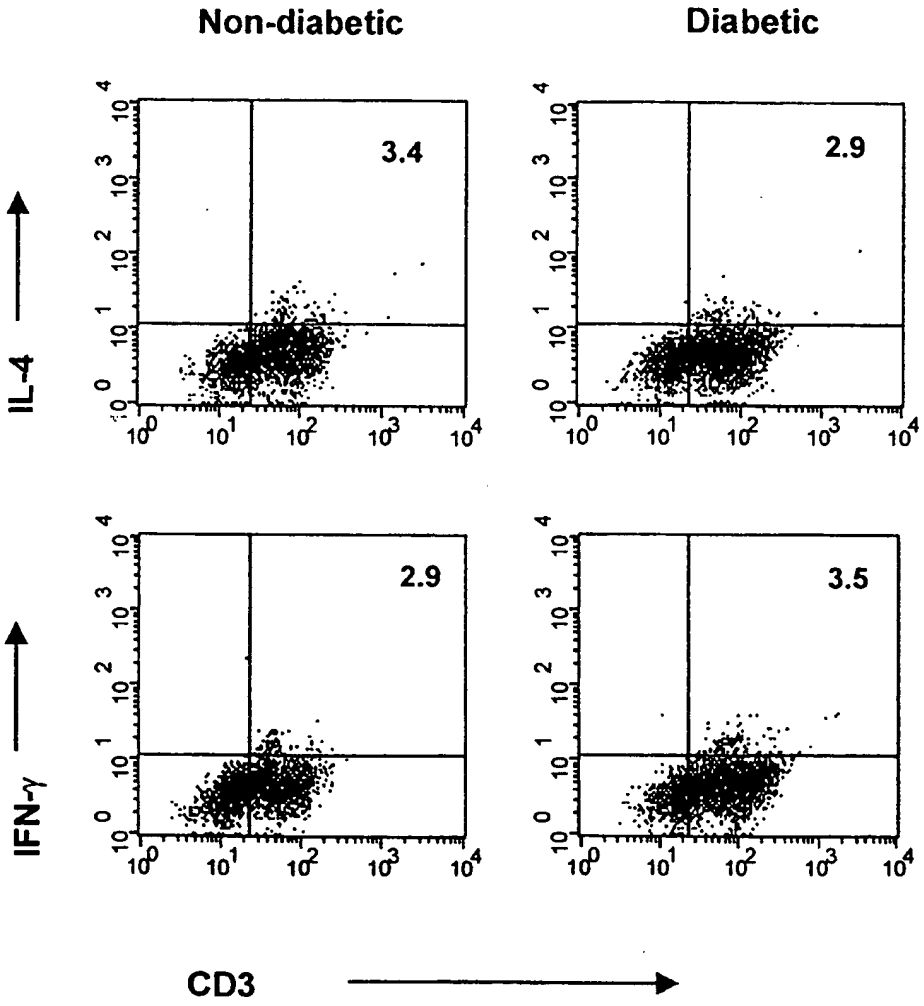


FIGURE 20 A

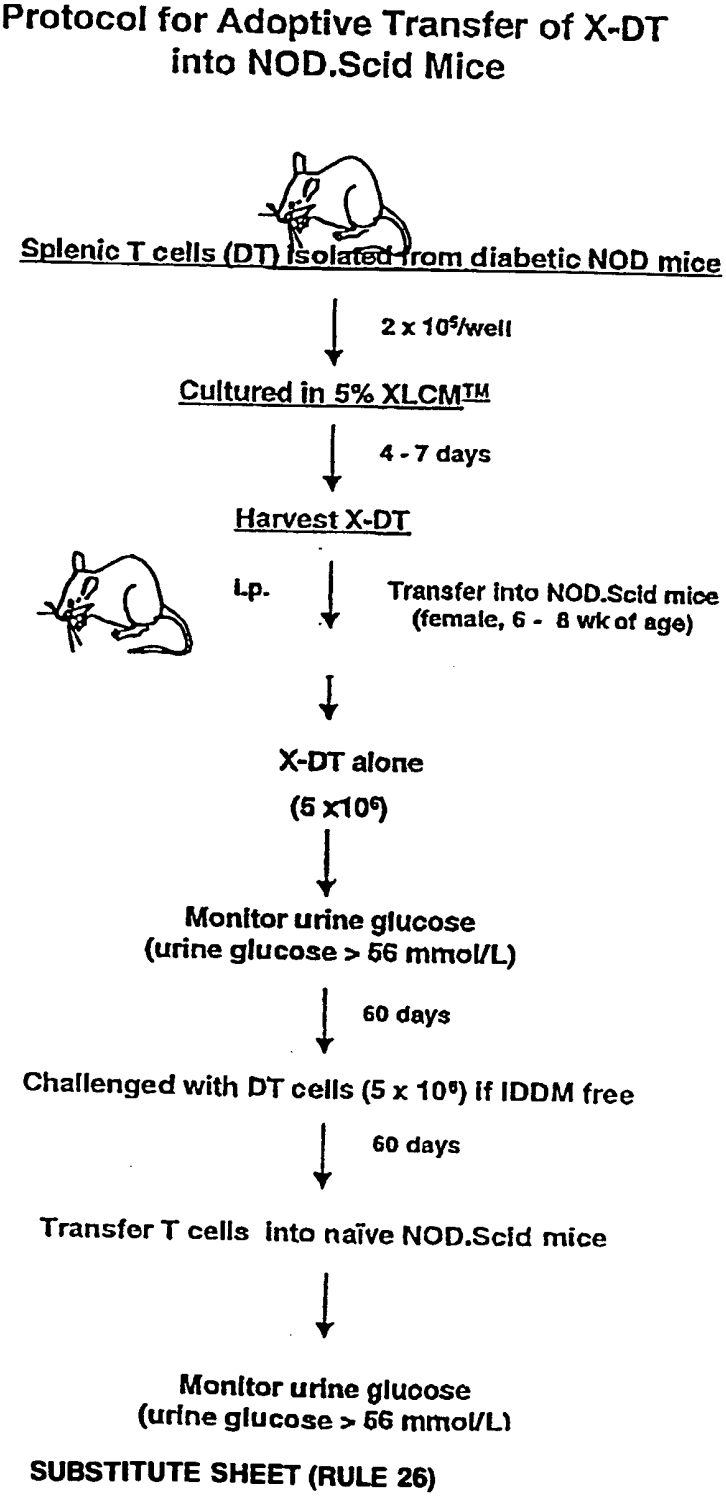

Protocol for Adoptive Transfer of X-DT into NOD.Scid Mice
Splenic T cells (DT) isolated from diabetic NOD mice
2 x 10⁵/well
Cultured in 5% XLCM™
4 - 7 days
Harvest X-DT
i.p.
Transfer into NOD.Scid mice (female, 6 - 8 wk of age)
X-DT alone
(5 x10⁶)
Monitor urine glucose
(urine glucose > 56 mmol/L)
60 days
Challenged with DT cells (5 x 10⁶) if IDDM free
60 days
Transfer T cells into naïve NOD.Scid mice
Monitor urine glucose
(urine glucose > 56 mmol/L)

Figure 20B

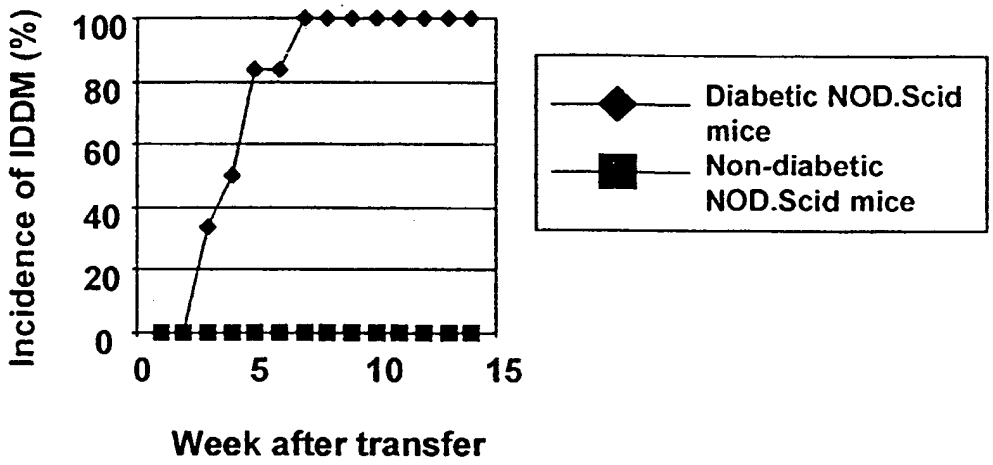

Incidence of IDDM (%)
100
80
60
40
20
0
0
5
10
15
Week after transfer
Diabetic NOD.Scid mice
Non-diabetic NOD.Scid mice

Figure 21A CD4+ X-DT Cells

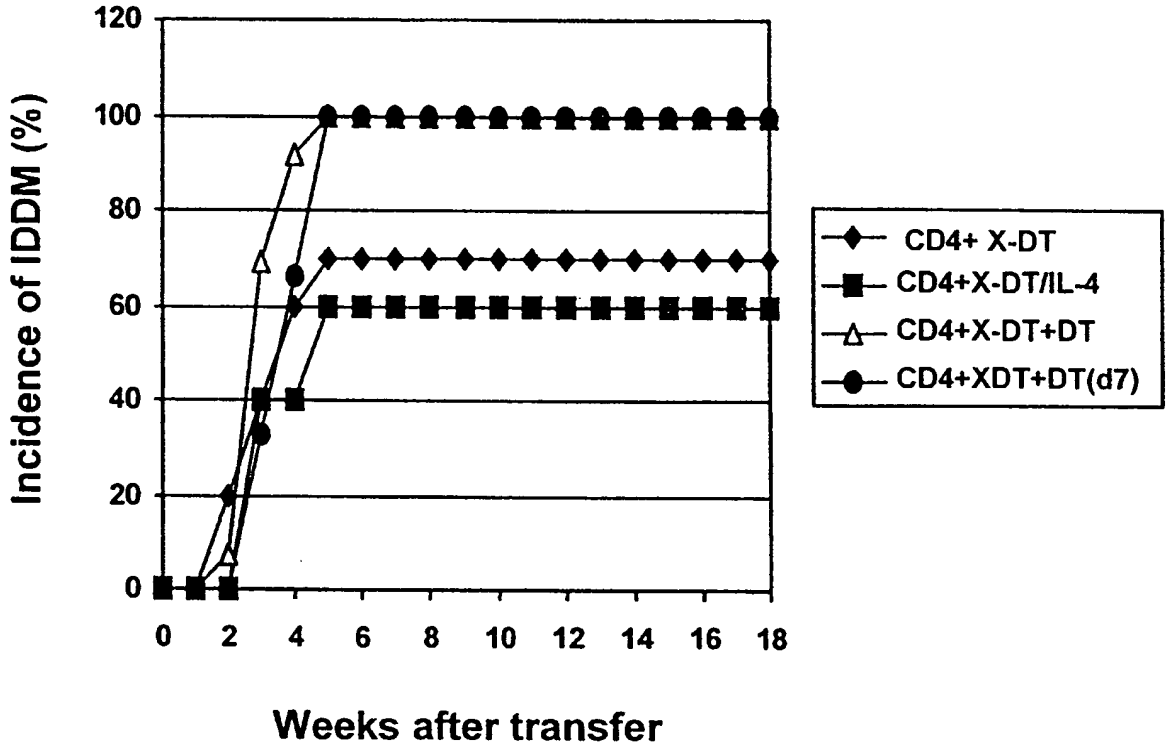
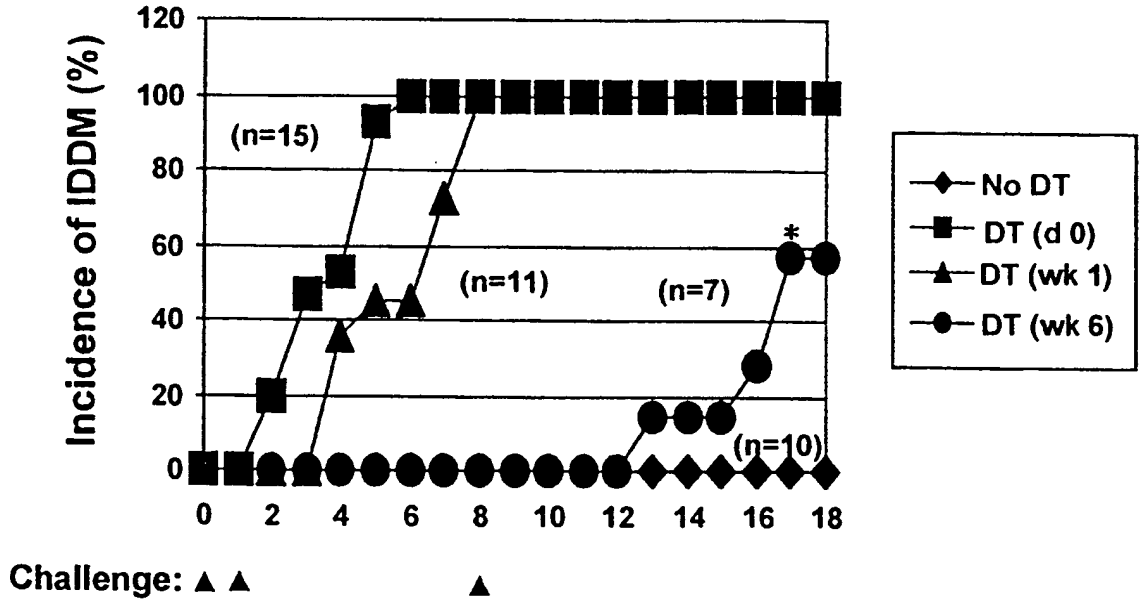


Figure 21B CD8+ X-DT Cells



INTERNATIONAL SEARCH REPORT

Inte. ..ional Application No
PCT/CA 99/00069

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MINGARI M C ET AL: "Development in vitro of human CD4 + thymocytes into functionally mature Th2 cells. Exogenous interleukin-12 is required for priming thymocytes to produce both Th1 cytokines and interleukin-10." EUROPEAN JOURNAL OF IMMUNOLOGY, (1996 MAY) 26 (5) 1083-7, XP002105035 see the whole document ---	1-30
A	CAMERON M J ET AL: "IL-4 prevents insulitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function." JOURNAL OF IMMUNOLOGY, (1997 NOV 15) 159 (10) 4686-92, XP002105036 see the whole document ---	1-30

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 June 1999	21/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. ..ional Application No
PCT/CA 99/00069

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SKEA, D. ET AL: "Large ex vivo expansion and reduced alloreactivity of umbilical cord blood T lymphocytes." BLOOD, (NOV. 15, 1997) VOL. 90, NO. 10 SUPPL. 1 PART 1, PP. 368A. MEETING INFO.: 39TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY SAN DIEGO, CALIFORNIA, USA DECEMBER 5-9, 1997, XP002105221 cited in the application see the whole document ---	1,2
P,X	LHOTAK, V. (1) ET AL: "Efficient ex vivo expansion of T cells from HIV-infected individuals using XLCMTM." BLOOD, (NOV. 15, 1998) VOL. 92, NO. 10 SUPPL. 1 PART 1-2, PP. 168A. MEETING INFO.: 40TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY MIAMI BEACH, FLORIDA, USA DECEMBER 4-8, 1998, XP002105037 see the whole document ---	1-13
P,X	ROBINSON, K. L. (1) ET AL: "Ex vivo expansion of umbilical cord blood (UCB) with XLCM leads to the preferential expansion, activation and maturation of CD4+ and CD8+ T lymphocytes: Development of UCB specific CTLs." BLOOD, (NOV. 15, 1998) VOL. 92, NO. 10 SUPPL. 1 PART 1-2, PP. 543A. MEETING INFO.: 40TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY MIAMI BEACH, FLORIDA, USA DECEMBER 4-8, 1998, XP002105038 see the whole document -----	1-13,18

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 99/ 00069

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 18-23 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.